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(54) Title: POLYNUCLEOTIDE VACCINE FORMULATIONS

(57) Abstract

The present invention relates to a novel vaccine formulation comprising nucleic acid molecules and a mineral–based adjuvant provided in a biologically effective concentration so as to improve induction of an immune response subsequent to vaccination which correlates to expression of one or more specific antigens encoded by the nucleic acid molecule.

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TITLE OF THE INVENTION
POLYNUCLEOTIDE VACCINE FORMULATIONS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of U.S. Application Serial No. 09/112,655, filed July 9, 1998, which is a continuation-in-part of U.S. Application Serial No. 09/023,834, filed February 13, 1998, which is a continuation-in-part of U.S. Provisional Application Serial No. 60/038,194, filed February 14, 1997.

STATEMENT REGARDING FEDERALLY-SPONSORED R&D

Not applicable.

REFERENCE TO MICROFICHE APPENDIX

Not applicable.

FIELD OF THE INVENTION

The present invention relates to novel vaccine formulations comprising nucleic acid molecules and an adjuvant which does not substantially bind the nucleic acid molecules, and their methods of use.

BACKGROUND OF THE INVENTION

A DNA vector containing a gene encoding a viral, bacterial, parasitic or tumor antigen has been shown to express that respective antigen in muscle cells and possibly other cell types subsequent to intramuscular injection. Such a naked DNA vector has come to be known as a polynucleotide vaccine (PNV) or DNA vaccine. The technique of using naked DNA as a prophylactic agent was reported in WO90/11092 (4 October 1990), in which naked polynucleotides were used to vaccinate vertebrates.

For example, both humoral and cell-mediated responses have been shown to occur when using DNA plasmid vectors encoding influenza antigens as a PNV, providing both homologous and cross-strain protection against a subsequent live virus challenge. The generation of both of these types of immune responses by a single vaccination approach offers a potential advantage over certain existing

vaccination strategies. The use of PNVs to generate antibodies may result in an increased duration of the antibody response, and may express an antigen having both the exact sequence of the clinically circulating strain of virus as well as the proper post-translational modifications and conformation of the native protein (vs. recombinant protein). The generation of CTL responses by this means offers the benefits of cross-strain protection without the use of a live potentially pathogenic vector or attenuated virus. For a review, see Donnelly, et al, 1997, *Life Sciences* 60: 163-172.

To date, PNVs have been in the form of DNA plasmid vectors which consist of a bacterial plasmid with a strong viral promoter, the DNA fragment containing an open reading frame which expresses the antigen of interest, and a polyadenylation/transcription termination sequence. The DNA plasmid vector is transformed into and grown in a bacterial host (such as *E. coli*) then purified and injected into the host in an aqueous solution. This PNV is taken up by a host cell (such as a muscle cell) wherein the antigen of interest is expressed. The plasmid is constructed so as to lack a eukaryotic origin of replication to limit host cell replication and/or host genome integration of the PNV construct.

Benvenisty and Reshef (1986, *Proc. Natl. Acad. Sci.*, 83: 9551-9555) showed expression of DNA co-precipitated with calcium phosphate and introduced into mice intraperitoneally into liver and spleen cells.

Subsequent studies by Wolff, et al. (1990, *Science* 247: 1465-1468) showed that the intramuscular injection of DNA expression vectors without $CaPO_4$ (e.g., in saline) in mice resulted in the uptake of DNA by the muscle cells and expression of the protein encoded by the DNA. The plasmids were maintained episomally and did not replicate Wolff, et al., 1992, *Human Mol. Genetics* 1:363-369). Persistent expression has been observed after intramuscular injection in skeletal muscle of rats, fish and primates, and cardiac muscle of rats.

Intravenous injection of a DNA:cationic liposome complex in mice was shown by Zhu et al. (1993, *Science* 261: 209-211) to result in systemic expression of a cloned transgene.

It has been shown that a PNV may be delivered to the target cell by particle bombardment, whereby the polynucleotide is adsorbed onto gold microprojectiles and delivered directly intracellularly by high velocity bombardment. This method has been used to induce an immune response to human growth hormone (Tang, et al., 1992, *Nature* 356: 152-154), influenza HA (Eisenbraun, et al., 1993,

DNA Cell Biol: 12: 791-797; Fynan, et al., 1993, *Proc. Natl. Acad. Sci.* 90: 11478-11482) and HIV gp120 (Eisenbraun, et al., 1993, *DNA Cell Biol*: 12: 791-797).

One major advantage purported of DNA vaccines is direct injection of the construct of interest in a saline or PBS solution without the addition of an adjuvant as seen with whole cell, acellular and subunit vaccines.

Adjuvants which have historically been used to enhance the immune response of classical whole cell, acellular and subunit vaccines include the mineral based compounds such as aluminum phosphate, aluminum hydroxide and calcium phosphate. These particular compounds are known in the art for a history of safe use as vaccine adjuvants, and are currently the only adjuvants approved for use in humans in the United States. Calcium phosphate is currently approved for use in humans in Europe. An aluminum phosphate adjuvant is actually amorphous aluminum hydroxyphosphate, $Al(OH)_m(PO_4)_n$ and an aluminum hydroxide adjuvant is actually an aluminum oxyhydroxide composition, AlO(OH). Aluminum phosphate is commercially available as an amorphous aluminum hydroxyphosphate gel (known as Adju-Phos®). These adjuvants have different charges at neutral pH, with AlO(OH) being positively charged and aluminum phosphate being negatively charged (see Gupta, et al., 1995, Ch.8 at page 231, in *Vaccine Design: The Subunit and Adjuvant Approach, Eds.* Powell and Newman, Plenum Press (New York and London). Vaccines containing $AlPO_4$ as an adjuvant are known to stimulate IL-4 and a T_H2-type of helper T cell response, as well as increasing levels of IgG1 and IgE antibodies (Vogel and Powell, 1995, Ch.7, in *Vaccine Design: The Subunit and Adjuvant Approach, Eds.* Powell and Newman, Plenum Press (New York and London) @ p. 142. Aluminum hydroxide is commercially available in crystalline form as aluminum oxyhydroxide (Alhydrogel®), and is also known as boehmite. Vaccines comprising AlO(OH) as an adjuvant also stimulate IL-4, T-helper-2 subsets, as well as increasing levels of IgG1 and IgE antibodies (Vogel and Powell, 1995, Ch.7, in *Vaccine Design: The Subunit and Adjuvant Approach, Eds.* Powell and Newman, Plenum Press (New York and London) @ p. 146.

It is also known in the art that preparations of both amorphous aluminum hydroxyphosphate gel and aluminum oxyhydroxide used in commercial vaccines vary. Shirodkar, et al. (1990, *Pharm. Res.* 7(12): 1282-1288) investigated nine commercially available aluminum-containing adjuvants by X-ray diffraction, infrared spectroscopy, electron microscopy and energy dispersive spectrometry. These authors reiterate that the commercially available form of aluminum phosphate

is an amorphous hydroxyphosphate and the aluminum hydroxide form is aluminum oxyhydroxide, or boehmite.

Effective adjuvanticity is known to be dependent on adsorption of the antigen of interest to an aluminum adjuvant. Studies suggest that electrostatic forces are paramount in effective absorption. Seeber, et al. (1991, *Vaccine* 9: 201-203) show that the importance of electrostatic forces is such that antigens with a high isoelectric point should be adsorbed to Adju-Phos® whereas antigens with a low isoelectric point may best be adsorbed to (Alhydrogel®).

Al-Shakhshir, et al. (1994, *Vaccine* 12(5): 472-474 show that protein adsorption to preformed aluminum adjuvants affects the surface charge characteristics of the adjuvant. Therefore, knowledge of both the adjuvant and protein surface properties are of importance in predicting the nature of a classical antigen-adjuvant vaccine formulation.

As noted above, calcium phosphate is another mineral salt which has been successfully used as an adjuvant to traditional protein vaccines. The use of calcium phosphate as an adjuvant is known and was first disclosed by Relyveld, et al. (1964, *Bull. WHO* 30: 321-325). The properties of a calcium phosphate adjuvant gel are controlled by the concentration of disodium hydrogen phosphate and calcium chloride utilized, as well the mixing rate (i.e., slower mixing rates resulting in a lower calcium to phosphate ratio). As with other adjuvants, binding to the antigen of interest is a prerequisite for enhanced immunogenicity.

Despite advances in the use of naked DNA vector-based vaccines, there is a distinct need for a pharmaceutical formulation which results in an enhanced immune response in a vertebrate host of interest. The present invention addresses this need by disclosing a DNA vaccine formulation comprising an adjuvant which does not substantially bind DNA and increases immunogenicity subsequent to vaccination of a vertebrate host.

SUMMARY OF THE INVENTION

The present invention relates to a novel vaccine formulation comprising nucleic acid molecules and an adjuvant provided in a biologically effective concentration so as to promote the effective induction of an immune response directed toward one or more specific antigens encoded by the nucleic acid molecule.

A particular embodiment of the present invention relates to a DNA vaccine formulation wherein the adjuvant comprises mineral-based particles which are negatively charged in the DNA suspension. These particles possess a sufficient negative charge as to substantially retard binding to the nucleic acid molecule of interest. Such a DNA-adjuvant composition will increase the immune response and may decrease nuclease digestion of the DNA vaccine, within the vertebrate host subsequent to immunization.

A preferred embodiment of the present invention relates to a DNA vaccine formulation which comprises a non-DNA binding mineral-based adjuvant generated from one or more forms of an aluminum phosphate-based adjuvant.

An especially preferred embodiment of the present invention relates to a DNA vaccine formulation wherein the aluminum phosphate-based adjuvant possesses a molar PO_4/Al ratio of approximately 0.9, including but not limited to Adju-Phos®.

Another embodiment of the present invention relates to a DNA vaccine formulation which comprises a non-DNA binding mineral-based adjuvant generated from one or more forms of a calcium phosphate-based adjuvant. DNA vaccines formulated with calcium phosphate increase antibody responses when the adjuvant is added at concentrations which do not result in a high percentage of bound DNA. In other words, calcium phosphate is an effective adjuvant for a DNA vaccine if the formulation contains a substantial amount of free DNA.

The nucleic acid molecule of the present invention may include a deoxyribonucleic acid molecule (DNA), such as genomic DNA and complementary DNA (cDNA) as well as a ribonucleic acid molecule (RNA). The nucleic acid molecules comprising the vaccine formulations of the present invention preferably do not show substantial binding to the chosen adjuvant. Of course, the skilled artisan will be aware that within any such vaccine formulation, the possibility remains that a measurable, but not biologically determinative, amount of nucleic acid molecules used in the present invention may bind to the chosen adjuvant.

The DNA construct may be delivered to the host in the form of a recombinant viral vector (including but in no way limited to a recombinant adenovirus vector, a recombinant adeno-associated vector, recombinant retrovirus vector, a recombinant Sindbis virus vector, and a recombinant alphavirus vector, all known in the art). The DNA construct may also be delivered via a recombinant

bacterial vector, such as recombinant BCG or Salmonella. Alternatively, the DNA may be associated with liposomes, such as lecithin liposomes or other liposomes known in the art, as a DNA-liposome mixture (see, for example, WO93/24640). However, a preferred vaccine formulation of the present invention comprises a non-viral DNA vector, most preferably a DNA plasmid-based vector. Standard recombinant DNA techniques for preparing and purifying DNA constructs are used to prepare the DNA polynucleotide constructs utilized in the exemplified PNV vaccine constructs disclosed throughout this specification.

Vaccine vectors for use in generating the vaccine formulations of the present invention, as well as practicing the related methods, include but are not necessarily limited to the DNA plasmid vectors V1, V1J, V1Jneo, VIJns, V1Jp, V1R and V1Jns-tPA.

The Example sections exemplify various polynucleotide vaccine constructs, such as a DNA plasmid vector expressing hemagglutinin (HA), a surface glycoprotein of influenza A, the nucleoprotein of influenza A, the HBsAg surface antigen from hepatitis B, as well as gp 120 and gag constructs from HIV. Therefore, it is evident that this specification gives excellent guidance to the skilled artisan to utilize the nucleic acid formulations of the present invention with an additional construction not expressly exemplified in the Example sections. Therefore, numerous other constructs representing different DNA constructs, modes of delivery, disease and antigen targets are envisioned for use in the vaccine formulations of the present invention. Examples of viral or bacterial challenges which may be amenable to either a prophylactic or therapeutic treatment include but are not limited to influenza, herpes simplex virus (HSV), human immunodeficiency virus (HIV), tuberculosis, human papilloma virus, hepatitis A, hepatitis B, and hepatitis C. It will also be within the scope of the present invention to provide prophylactic or, most likely, therapeutic treatment for non-infectious diseases, such as cancer, autoimmune disorders, and various allergies. Additionally, it will be within the purview of the skilled artisan to utilize the formulations of the present invention for any number of veterinary applications, including but not limited to rabies, distemper, foot and mouth disease, anthrax, bovine herpes simplex and bovine tuberculosis.

The present invention also relates to methods of generating an immune response in a vertebrate host, such as a human, by administering the DNA vaccine formulations of the present invention.

The term "polynucleotide" as used herein is a nucleic acid which contains essential regulatory elements such that upon introduction into a living, vertebrate cell, it is able to direct the cellular machinery to produce translation products encoded by the genes comprising the polynucleotide.

The term "substantially retard binding", "does not substantially bind", or similar language as used herein refers the concept that a small proportion of the nucleic acid may in fact bind adjuvant within the vaccine formulation. However, any such bound material does not affect the intended biological consequence of the vaccine formulations of the present invention. Any decrease in biological activity in response to such binding may easily be overcome by adjusting slightly upward the dosage given to the vertebrate host.

The term "promoter" as used herein refers to a recognition site on a DNA strand to which the RNA polymerase binds. The promoter forms an initiation complex with RNA polymerase to initiate and drive transcriptional activity. The complex can be modified by activating sequences termed "enhancers" or inhibiting sequences termed "silencers."

The term "leader" as used herein refers to a DNA sequence at the 5' end of a structural gene which is transcribed along with the gene. The leader usually results in the protein having an N-terminal peptide extension sometimes called a pro-sequence. For proteins destined for either secretion to the extracellular medium or a membrane, this signal sequence, which is generally hydrophobic, directs the protein into endoplasmic reticulum from which it is discharged to the appropriate destination.

The term "intron" as used herein refers to a portion or portions of a gene which does not encode a portion of the gene product. Introns from the precursor RNA are excised, wherein the resulting mRNA translates the respective protein.

The term "cassette" refers to the sequence of the present invention which contains the nucleic acid sequence which is to be expressed. The cassette is similar in concept to a cassette tape. Each cassette will have its own sequence. Thus by interchanging the cassette the vector will express a different sequence. Because of the restrictions sites at the 5' and 3' ends, the cassette can be easily inserted, removed or replaced with another cassette.

The term "3' untranslated region" or "3' UTR" refers to the sequence at the 3' end of a structural gene which is usually transcribed with the gene. This 3' UTR region usually contains the poly A sequence. Although the 3' UTR is transcribed from the DNA it is excised before translation into the protein.

The term "Non-Coding Region" or "NCR" refers to the region which is contiguous to the 3' UTR region of the structural gene. The NCR region contains a transcriptional termination signal.

The term "vector" refers to some means by which DNA fragments can be introduced into a host organism or host tissue. There are various types of vectors which include but are not limited to recombinant vectors, including DNA plasmid vectors, viral vectors such as adenovirus vectors, retrovirus vectors and adeno-associated virus vectors, as well as bacteriophage vectors and cosmid vectors.

The term "biologically effective amount" means sufficient PNV is injected to produce the adequate levels of the polypeptide. One skilled in the art recognizes that this level may vary.

The term "gene" refers to a segment of nucleic acid which encodes a discrete polypeptide.

The terms "pharmaceutical" and "vaccine" are used interchangeably to indicate compositions useful for inducing immune responses.

BRIEF DECRIPTION OF THE DRAWINGS/FIGURES.

Figure 1A and Figure 1B show the effect of aluminum phosphate on the generation of anti-HA antibody titers in mice at 4 weeks post 1 injection (Figure 1A) and 8 weeks post 1 injection (Figure 1B) at DNA HA doses of 0.5 μg and 10 μg.

Figure 2A and Figure 2B show a time course measurement of anti-HA antibody titers in mice after a single innoculation of FR-9502 HA DNA (A/Georgia/93), with (●) and without (■) aluminum phosphate injection at DNA HA doses of 0.5 μg (Figure 2A) and 10 μg (Figure 2B).

Figure 3A and Figure 3B show that a range of DNA doses enhance the immune response in mice, as measured by anti-HA antibody production after a single innoculation of FR-9502 HA DNA (A/Georgia/93) as measured by HI titer (Figure 3A) or ELISA titer (Figure 3B).

Figure 4 shows the enhancement of anti-NP antibody responses in mice after innoculation with NP plasmid DNA with or without aluminum phosphate at DNA doses of 5 μg and 50 μg at 6 weeks post 1 injection and 3 weeks post 2 injections.

Figure 5A (IL-2), Figure 5B (INF-γ), Figure 5C (IL-4) and Figure 5D (IL-10) show the effect of aluminum phosphate on respective cytokine secretion from

antigen restimulation spleen cells of NP plasmid DNA inoculated mice (6 weeks post 1 injection and 3 weeks post 2 injection) at DNA doses of 5 mcg and 50 mcg with one, two or three injections.

Figure 6A - Figure 6D show the effect of aluminum phosphate on the cytotoxic T lymphocyte response after a single innoculation of NP plasmid DNA innoculation in mice: Figure 6A (5 μg DNA, 6 weeks post injection, flu-infected target cells); Figure 6B (5 μg DNA, 6 weeks post injection, peptide pulsed target cells); Figure 6C (50 μg DNA, 6 weeks post injection, flu-infected target cells); and, Figure 6D (50 μg DNA, 6 weeks post injection, peptide-pulsed target cells).

Figure 7 shows the effect of aluminum phosphate on the antibody response to inoculation of mice with a DNA vaccine (V1R.S) encoding hepatitis B surface antigen. A 1μg dose of Recombivax HB® was compared for immunogenicity with the V1R.S vaccine injected with or without 45 μg of aluminum phosphate (Adju-Phos®). Mice were injected at day 0 and day 42 with Recombivax HB® (●), 100 μg HBV DNA with adjuvant (◆), 100 μg HBV DNA without adjuvant (■), or 1μg of HBsAg (protein) without adjuvant (◊).

Figure 8 shows the effect of HBV DNA vaccine (V1R.S) dosing with and without adjuvant on HBsAg antibody production six weeks after a single injection of mice. Forty five μg of aluminum phosphate (AdjuPhos®) or aluminum hydroxyphosphate was added with 1 μg, 10 μg and 100 μg HBV DNA with and without adjuvant.

Figure 9 shows the effect of a second dose at day 42 (bleed at day 63) for the dosing effects disclosed for Figure 8.

Figure 10 shows the induction of a CTL response in response to DNA vaccination with V1R.S for a formulation with and without an aluminum phosphate adjuvant (45 μg/100 μl sample).

Figure 11 shows the effect of aluminum phosphate or calcium phosphate on the gp120 and gag antibody response after inoculation of mice with a HIV env/gag DNA plasmid construct, as measured by an ELISA assay.

Figure 12A and Figure 12B show a time course measurement of anti-DNA antibody titers in rhesus monkeys after a single inoculation with FR-9502 DNA as measured by geometric mean HI titer (Figure 12A) or ELISA (Figure 12B).

Figure 13A and Figure 13B show that BALB/c mice were immunized with 10 μg of HBV DNA vaccine ± aluminum phosphate on day 0 and 21. Eight days later, spleen cells were harvested and tested individually in the ELISPOT assay

for IFN-γ (Figure 13 A) or IL-2 (Figure 13B). The IFN-γ response was elicited by overnight culture with 5-fold dilutions of HBs peptide [28-39], and the IL-2 response was stimulated by culture with 5-fold dilutions of HBs peptide [146-160]. The results are expressed as the mean ± SE SFC/10^6 spleen cells with n = 5 mice per group.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a novel vaccine formulation comprising nucleic acid molecules and an adjuvant provided in a biologically effective concentration so as to promote the effective induction of an immune response directed toward one or more specific antigens encoded by the nucleic acid molecule.

A particular embodiment of the present invention relates to a DNA vaccine formulation wherein the adjuvant comprises mineral-based particles which are negatively charged in the DNA suspension. These particles possess a sufficient negative charge as to substantially retard binding to the nucleic acid molecule of interest. Such a DNA-adjuvant composition will increase the immune response and may decrease nuclease digestion of the DNA vaccine, within the vertebrate host subsequent to immunization.

A preferred embodiment of the present invention relates to a DNA vaccine formulation which comprises a non-DNA binding mineral adjuvant generated from one or more forms of an aluminum phosphate-based adjuvant. The term "aluminum phosphate" is oftentimes used in the art to describe members of a continuous series of aluminum hydroxyphosphate compositions in which the molar PO_4/Al ratio ranges from about 0.3 to about 0.9 (Hem and White, 1995, Ch. 9, in *Vaccine Design: The Subunit and Adjuvant Approach, Eds.* Powell and Newman, Plenum Press (New York and London). As noted throughout this specification, numerous conditions exist to generate the various aluminum hydroxyphosphate gels for use in the vaccine formulations of the present invention. For instance, the skilled artisan will note that Hem and White, *supra* at page 244-255 describe specific factors which will affect the surface charge of the resulting adjuvant. Hem and White state that generating an aluminum phosphate adjuvant with aluminum salts having a weak affinity for aluminum, such as aluminum chloride, will result in an adjuvant with a higher phosphate content than using an aluminum salt with a higher affinity toward aluminum, such as a sulfate

anion. It will also be possible to affect the final adjuvant composition by controlling the speed of mixing, the speed and conditions for adjuvant precipitation, heating, and other physical manipulations known to the skilled artisan. In other words, numerous strategies are known and are available to generate an aluminum phosphate-based adjuvant which has a molar PO_4/Al ratio such that the adjuvant will carry a net negative charge and would be expected to not substantially bind to DNA in the vaccine formulations of the present invention.

An especially advantageous aluminum phosphate adjuvant, albeit by no means a limiting one, is a substantially negatively charged aluminum phosphate based adjuvant wherein the molar PO_4/Al is approximately 0.9. For example, Adju-Phos® is a commercially available form of amorphous aluminum hydroxyphosphate gel which represents an especially preferred adjuvant for use in the DNA vaccine formulations of the present invention. This preference depends on the fact that the amorphous aluminum hydroxyphosphate Adju-Phos® is comprised of negatively charged, micron-sized particles which do not substantially bind DNA in the formulations of the present invention.

The skilled artisan will be aware that the nature of the adjuvant and its ability to bind classic antigens is effected by the conditions whereby the adjuvant is initially precipitated, the precipitation conditions, pH, temperature, and ionic strength. These same type of component manipulations will be available to the skilled artisan to alter the surface charge of various aluminum phosphate-based adjuvants to create an adjuvant surface charge conducive to use in the DNA vaccine formulations of the present invention. Therefore, it will be within the purview of the skilled artisan to take an aluminum hydroxyphosphate adjuvant with, say for example, a molar PO_4/Al ratio closer to 0.3, and alter the conditions of the vaccine formulation such that the manipulated adjuvant will possess a negative surface charge which substantially retards DNA binding. It is also within the boundary of the present invention to manipulate an aluminum hydroxide adjuvant (such as Alhydrogel®) by manipulating conditions including but not limited to adjuvant precipitation conditions, formulation buffer conditions, pH, temperature, and ionic strength. The goal of such an adjuvant manipulation will be to generate an adjuvant with a negatively charged surface such that adjuvant-DNA binding will be substantially prohibited. Therefore, the skilled artisan will understand after review of this specification that negatively charged

adjuvants which inhibit substantial adjuvant-DNA binding may be generated by any number of procedures which are well known and readily available.

Also, the skilled artisan will be aware that non-commercial sources of aluminum phosphate-based adjuvants may be formed for use in the DNA vaccine formulations of the present invention. Such methods include but are in no way limited to mixing aluminum chloride and trisodium phosphate to generate aluminum phosphate. Once again, the skilled artisan is aware that the nature of the adjuvant and its ability to bind to classic antigens is affected by numerous variables, including but not limited to adjuvant precipitation conditions, formulation buffer conditions, pH, temperature, and ionic strength. These same type of component manipulations will be available to the skilled artisan to alter the surface charge of various non-commercial forms of aluminum hydroxyphosphate adjuvants to create an adjuvant surface charge conducive to use in the DNA vaccine formulations of the present invention. More specifically, these negatively charged adjuvants will inhibit substantial adjuvant-DNA binding and will promote the expected immune response upon vertebrate host vaccination. The present invention also relates to DNA vaccine formulations which comprise a calcium phosphate-based adjuvant. A calcium phosphate adjuvant gel may be generated by known methods of mixing disodium hydrogen phosphate and calcium chloride. As noted within this specification for aluminum phosphate-based adjuvants, a preferred calcium phosphate adjuvant for the vaccine formulations of the present invention is an adjuvant with a sufficient negative surface charge as to substantially retard binding to the DNA construct of interest. Data is presented in Example section 10 showing that calcium phosphate is an effective adjuvant for DNA vaccines so long a there remains within the formulation a substantial amount of free (i.e., unbound) DNA. It will be within the purview of the artisan to determine an optimal adjuvant and DNA dose or dose range so as to maximize the adjuvant effect while a biologically active amount of free DNA remains in the formulation. The DNA vaccine formulations of the present invention will contain from about 1 to about 20,000 mcg of aluminum or calcium (in an adjuvanted form such as aluminum phosphate, calcium phosphate), preferably from about 10 to about 10,000 mcg and most preferably from about 25 to about 2,500 mcg. Particular formulations may require particular amounts within these ranges, for example, about 20, 45, 90, 100, 200, 450, 750, 900, 1,500, 2,500, 3,500 mcg, 10,000 mcg, etc., or other amounts not listed here, may be used.

It is noted that a majority of data reported for mice in the Example sections utilize a 100 µl injection of the DNA vaccine formulation. Therefore, a formulation comprising aluminum at 450 mcg/mL results in a 45 mcg dose of aluminum, and is referred throughout the specification as an adjuvant dose, such as 450 mcg/mL of Adju-Phos®. It should be noted that the term "mcg" is used interchangebly with "µg" throughout this specification to represent the unit of measurement, microgram.

The nucleic acid molecule of the present invention may include a deoxyribonucleic acid molecule (DNA), such as genomic DNA and complementary DNA (cDNA) as well as a ribonucleic acid molecule (RNA). The DNA of the present invention is associated, but preferably does not bind, a mineral-based adjuvant.

The DNA construct may be delivered to the host in the form of a recombinant viral vector (including but in no way limited to a recombinant adenovirus vector, a recombinant adeno-associated vector, recombinant retrovirus vector, a recombinant Sindbis virus vector, and a recombinant alphavirus vector, all known in the art). The DNA construct may also be delivered via a recombinant bacterial vector, such as recombinant BCG or Salmonella. Alternatively, the DNA may be associated with lipids to form DNA-lipid complexes or with lipids in the form of liposomes, such as lecithin liposomes or other liposomes known in the art, to form DNA-liposome mixture (see, for example, WO93/24640.

However, a preferred vaccine formulation of the present invention comprises a non-viral DNA vector, most preferably a DNA plasmid-based vector. Standard recombinant DNA techniques for preparing and purifying DNA constructs are used to prepare the DNA polynucleotide constructs utilized in the exemplified PNV vaccine constructs disclosed throughout this specification. A gene of interest is ligated into an expression vector which has been optimized for polynucleotide vaccinations. Extraneous DNA is at least partially removed, leaving essential elements such as a transcriptional promoter, immunogenic epitopes, transcriptional terminator, bacterial origin of replication and antibiotic resistance gene.

The amount of expressible DNA to be introduced to a vaccine recipient will depend on the strength of the transcriptional and translational promoters used in the DNA construct, and on the immunogenicity of the expressed gene product. In general, an immunologically or prophylactically effective dose of about 1 µg to greater than about 5 mg, and preferably about 10 µg to 2 mg is administered

directly into muscle tissue. Subcutaneous injection, intradermal introduction, impression through the skin, and other modes of administration such as intraperitoneal, intravenous, inhalation and oral delivery are also contemplated. It is also contemplated that boosfer vaccinations are to be provided. In this case, it is desirable for the DNA to be in a physiologically acceptable solution, such as, but not limited to, sterile saline or sterile buffered saline, taking into consideration the effect that pH, buffer conditions and ionic charge may have on the net surface charge of the mineral-based adjuvant used to formulate the DNA vaccines of the present invention.

Vaccine vectors for use in practicing the present invention include but are not necessarily limited to the DNA plasmid vectors V1, V1J, V1R, V1Jp, V1Jneo, VIJns, and V1Jns-tPA,

Vaccine vector V1 was constructed from pCMVIE-AKI-DHFR (Whang et al., 1987, *J. Virol.* 61: 1796). The AKI and DHFR genes were removed by cutting the vector with EcoRI and self-ligating. This vector does not contain intron A in the CMV promoter, so it was added as a PCR fragment that had a deleted internal SacI site [at 1855 as numbered in Chapman, et al., 1991, *Nuc. Acids Res.* 19: 3979). The template used for the PCR reactions was pCMVintA-Lux, made by ligating the HindIII and NheI fragment from pCMV6a120 (see Chapman et al., *ibid.*), which includes hCMV-IE1 enhancer/promoter and intron A, into the HindIII and XbaI sites of pBL3 to generate pCMVIntBL. The 1881 base pair luciferase gene fragment (HindIII-SmaI Klenow filled-in) from RSV-Lux (de Wet et al., 1987, *Mol. Cell Biol.* 7: 725) was ligated into the SalI site of pCMVIntBL, which was Klenow filled-in and phosphatase treated. The primers that spanned intron A are: 5' primer: 5'-CTATATAAGCAGAGCTCGTTTAG-3' (SEQ ID NO:1); 3' primer: 5'-GTAGCAAAGATCTAAGGACGGTGACTGCAG-3' (SEQ ID NO:2). The primers used to remove the SacI site are:
sense primer, 5'-GTATGTGTCTGAAAATGAGCGTGGAGATTGGGC TCGCAC-3' (SEQ ID NO:3) and the antisense primer,
5'-GTGCGAGCCCAATCTCCACGCTCATTTTCAGACACATAC-3' (SEQ ID NO:4). The PCR fragment was cut with Sac I and Bgl II and inserted into the vector which had been cut with the same enzymes.

A V1J expression vector may be generated to remove the promoter and transcription termination elements from vector V1 in order to place them within a more defined context, create a more compact vector, and to improve plasmid purification yields. V1J is derived from vectors V1 and pUC18, a commercially

available plasmid. V1 was digested with SspI and EcoRI restriction enzymes producing two fragments of DNA. The smaller of these fragments, containing the CMVintA promoter and Bovine Growth Hormone (BGH) transcription termination elements which control the expression of heterologous genes, was purified from an agarose electrophoresis gel. The ends of this DNA fragment were then "blunted" using the T4 DNA polymerase enzyme in order to facilitate its ligation to another "blunt-ended" DNA fragment. pUC18 was chosen to provide the "backbone" of the expression vector. It is known to produce high yields of plasmid, is well-characterized by sequence and function, and is of small size. The entire *lac* operon was removed from this vector by partial digestion with the HaeII restriction enzyme. The remaining plasmid was purified from an agarose electrophoresis gel, blunt-ended with the T4 DNA polymerase treated with calf intestinal alkaline phosphatase, and ligated to the CMVintA/BGH element described above. Plasmids exhibiting either of two possible orientations of the promoter elements within the pUC backbone were obtained. One of these plasmids gave much higher yields of DNA in *E. coli* and was designated V1J. This vector's structure was verified by sequence analysis of the junction regions and was subsequently demonstrated to give comparable or higher expression of heterologous genes compared with V1.

Construction of the V1Jneo expression vector requires removal of the amp^r gene used for antibiotic selection of bacteria harboring V1J because ampicillin may not be desirable in large-scale fermenters. The amp^r gene from the pUC backbone of V1J was removed by digestion with SspI and Eam1105I restriction enzymes. The remaining plasmid was purified by agarose gel electrophoresis, blunt-ended with T4 DNA polymerase, and then treated with calf intestinal alkaline phosphatase. The commercially available kan^r gene, derived from transposon 903 and contained within the pUC4K plasmid, was excised using the PstI restriction enzyme, purified by agarose gel electrophoresis, and blunt-ended with T4 DNA polymerase. This fragment was ligated with the V1J backbone and plasmids with the kan^r gene in either orientation were derived which were designated as V1Jneo #'s 1 and 3. Each of these plasmids was confirmed by restriction enzyme digestion analysis, DNA sequencing of the junction regions, and was shown to produce similar quantities of plasmid as V1J. Expression of heterologous gene products was also comparable to V1J for these V1Jneo vectors. V1Jneo#3, referred to as V1Jneo hereafter, was selected which contains the kan^r gene in the same orientation as the amp^r gene in V1J as the expression construct.

The expression vector VIJns was generated by adding an SfiI site to V1Jneo to facilitate integration studies. A commercially available 13 base pair SfiI linker (New England BioLabs) was added at the KpnI site within the BGH sequence of the vector. V1Jneo was linearized with KpnI, gel purified, blunted by T4 DNA polymerase, and ligated to the blunt SfiI linker. Clonal isolates were chosen by restriction mapping and verified by sequencing through the linker. The new vector was designated V1Jns. Expression of heterologous genes in V1Jns (with SfiI) was comparable to expression of the same genes in V1Jneo (with KpnI).

The DNA vaccine vector V1Jns-tPA was constructed in order to provide an heterologous leader peptide sequence to secreted and/or membrane proteins. Plasmid V1Jns was modified to include the human tissue-specific plasminogen activator (tPA) leader. Two synthetic complementary oligomers were annealed and then ligated into V1Jn which had been BglII digested. The sense and antisense oligomers were 5'-GATCACCATGGATGCAATGAAGAGAGGGCTC TGCTGTGTGCTGCTGCTGTGTGGAGCAGTCTTCGTTTCGCCCAGCGA-3' (SEQ ID NO:5); and, 5'-GATCTCGCTGGGCGAAACGAAGA CTGCTCCACACAGCAGCAGCACACAGCAGAGCCCTCTCTTCATTGCATCC ATGGT-3' (SEQ ID NO:6). The Kozak sequence is underlined in the sense oligomer. These oligomers have overhanging bases compatible for ligation to BglII-cleaved sequences. After ligation the upstream BglII site is destroyed while the downstream BglII is retained for subsequent ligations. Both the junction sites as well as the entire tPA leader sequence were verified by DNA sequencing. Additionally, in order to conform with the consensus optimized vector V1Jns (=V1Jneo with an SfiI site), an SfiI restriction site was placed at the KpnI site within the BGH terminator region of V1Jn-tPA by blunting the KpnI site with T4 DNA polymerase followed by ligation with an SfiI linker (catalogue #1138, New England Biolabs). This modification was verified by restriction digestion and agarose gel electrophoresis.

Yet another DNA vaccine vector, V1R, may be utilized to practice the present invention. This DNA vaccine vector is a derivative of V1Jns. This vector is useful to obtain a minimum-sized vaccine vector without unneeded DNA sequences, which still retained the overall optimized heterologous gene expression characteristics and high plasmid yields that V1J and V1Jns afford. It was determined that (1) regions within the pUC backbone comprising the *E. coli* origin of replication could be removed without affecting plasmid yield from bacteria; (2) the 3'-region of the *kan*^{r} gene following the kanamycin open reading frame could be removed if a bacterial

terminator was inserted in its place; and, (3) ~300 bp from the 3'- half of the BGH terminator could be removed without affecting its regulatory function (following the original KpnI restriction enzyme site within the BGH element). V1R was constructed by using PCR to synthesize three segments of DNA from V1Jns representing the CMVintA promoter/BGH terminator, origin of replication, and kanamycin resistance elements, respectively. Restriction enzymes unique for each segment were added to each segment end using the PCR oligomers: SspI and XhoI for CMVintA/BGH; EcoRV and BamHI for the *kan* ^{r} gene; and, BclI and SalI for the *ori* ^{r}. These enzyme sites were chosen because they allow directional ligation of each of the PCR-derived DNA segments with subsequent loss of each site: EcoRV and SspI leave blunt-ended DNAs which are compatible for ligation while BamHI and BclI leave complementary overhangs as do SalI and XhoI. After obtaining these segments by PCR each segment was digested with the appropriate restriction enzymes indicated above and then ligated together in a single reaction mixture containing all three DNA segments. The 5'-end of the *ori* ^{r} was designed to include the T2 rho independent terminator sequence that is normally found in this region so that it could provide termination information for the kanamycin resistance gene. The ligated product was confirmed by restriction enzyme digestion (>8 enzymes) as well as by DNA sequencing of the ligation junctions. DNA plasmid yields and heterologous expression using viral genes within V1R appear similar to V1Jns. The net reduction in vector size achieved was 1346 bp (V1Jns = 4.86 kb; V1R = 3.52 kb). PCR oligomer sequences used to synthesize V1R (restriction enzyme sites are underlined and identified in brackets following sequence) are as follows: (1) 5'-GGTACA AATA TTGGCTATTGGCCATTGCATACG-3' (SEQ ID NO:7) [SspI]; (2) 5'-CCACATCTCGAGGAACCGGGTCAATTCTTCAGCACC-3' (SEQ ID NO:8) [XhoI] (for CMVintA/BGH segment); (3) 5'-GGTACAGAT ATCGGAAAGCCACGTTGTGTCTCAAAATC-3' (SEQ.ID NO:9) [EcoRV]; (4) 5'-CACATGGATCCGTAATGCTCTGCCAGTGTT ACAACC-3' (SEQ ID NO:10) [BamHI], (for kanamycin resistance gene segment) (5) 5'-GGTACATGATCACGTAGAAAAGATCAAAGG ATCTTCTTG-3' (SEQ ID NO:11) [BclI]; (6) 5'-CCACATGTCGACCCG TAAAAAGGCCGCGTTGCTGG-3' (SEQ ID NO:12): [SalI], (for *E. coli* origin of replication).

The Example sections exemplify various polynucleotide vaccine constructs, such as a DNA plasmid vector expressing hemagglutinin (HA), a surface

glycoprotein of influenza A, the nucleoprotein of influenza A, the HBsAg surface antigen from hepatitis B, as well as gp 120 and gag constructs from HIV. Therefore, it is evident that this specification gives excellent guidance to the skilled artisan to utilize the nucleic acid formulations of the present invention with an additional construction not expressly exemplified in the Example sections. Therefore, it will be within the purview of the skilled artisan to grasp the teachings of this specification so as to use any variation in regard to the type of nucleic acid molecule used (such as DNA plasmid, recombinant viral vectors such as adenovirus, adeno-associated virus, retrovirus) as well as the type of viral or bacterial antigen expressed. Examples of viral or bacterial challenges which may be amenable to either a prophylactic or therapeutic treatment include but are not limited to influenza, herpes simplex virus (HSV), human immunodeficiency virus (HIV), tuberculosis, human papilloma virus, hepatitis A, hepatitis B, and hepatitis C. It will also be within the scope of the present invention to provide prophylactic or therapeutic treatment for non-infectious diseases, such as cancer, autoimmune disorders, and various allergies. This approach to vaccination will be applicable to tumors as well as infectious agents, since the $CD8^+$ CTL response is important for both pathophysiological processes (Tanaka, et al., 1988, *Annu. Rev. Immunol.* 6: 359). Therefore, eliciting an immune response against a protein crucial to the transformation process may be an effective means of cancer protection or immunotherapy. The generation of high titer antibodies against expressed proteins after injection of viral protein and human growth hormone DNA suggests that this is a facile and highly effective means of making vaccines that induce, either separately or in combination with other vectors, antibody and/or CTL responses. The DNA vaccine formulations of the present invention will also be useful for any number of veterinary applications, including but not limited to rabies, distemper, foot and mouth disease, anthrax, bovine herpes simplex and bovine tuberculosis.

An improved HSV polynucleotide vaccine formulation of the present invention will comprise a nucleic acid vector encoding an HSV antigen of interest, including but not limited to gB, gD, ΔgB (encoding the amino-terminal 707 aa of HSV-2 gB) and ΔgD, alone or in combination.

The vaccine formulations of the present invention may also be directed to the prophylactic treatment of human immunodeficiency virus-1 (HIV-1). It is well known that HIV-1 is the etiological agent of acquired human immune deficiency syndrome (AIDS) and related disorders. HIV-1 is an RNA virus of the Retroviridae

family and exhibits the 5'LTR-*gag-pol-env*-LTR3' organization of all retroviruses. In addition, HIV-1 comprises a handful of genes with regulatory or unknown functions, including the tat and *rev* genes. The *env* gene encodes the viral envelope glycoprotein that is translated as a 160-kilodalton (kDa) precursor (gp160) and then cleaved by a cellular protease to yield the external 120-kDa envelope glycoprotein (gp120) and the transmembrane 41-kDa envelope glycoprotein (gp41). Gp120 and gp41 remain associated and are displayed on the viral particles and the surface of HIV-infected cells. Gp120 binds to the CD4 receptor present on the surface of helper T-lymphocytes, macrophages and other target cells. After gp120 binds to CD4, gp41 mediates the fusion event responsible for virus entry.

Infection begins when gp120 on the viral particle binds to the CD4 receptor on the surface of T4 lymphocytes or other target cells. The bound virus merges with the target cell and reverse transcribes its RNA genome into the double-stranded DNA of the cell. The viral DNA is incorporated into the genetic material in the cell's nucleus, where the viral DNA directs the production of new viral RNA, viral proteins, and new virus particles. The new particles bud from the target cell membrane and infect other cells.

Expression of HIV late genes such as *env* and *gag* is *rev*-dependent and requires that the *rev* response element (RRE) be present on the viral gene transcript. A secreted form of gp120 can be generated in the absence of *rev* by substitution of the gp120 leader peptide with a heterologous leader such as from tPA (tissue-type plasminogen activator), and preferably by a leader peptide such as is found in highly expressed mammalian proteins such as immunoglobulin leader peptides. A tPA-gp120 chimeric gene cloned into V1Jns efficiently expresses secreted gp120 in a transfected human rhabdomyosarcoma cell line. Monocistronic gp160 does not produce any protein upon transfection without the addition of a *rev* expression vector. Representative construct components include but are not limited to tPA-gp120$_{MN}$, gp160$_{IIIB}$, *gag*$_{IIIB}$: for anti-*gag* CTL, tPA-gp120$_{IIIB}$, tPA-gp140, and tPA-gp160 with structural mutations: V1, V2, and/or V3 loop deletions or substitutions.

The protective efficacy of polynucleotide HIV immunogens against subsequent viral challenge is demonstrated by immunization with the non-replicating plasmid DNA. This is advantageous since no infectious agent is involved, assembly of virus particles is not required, and determinant selection is permitted. Furthermore, because the sequence of *gag* and protease and several of the other viral gene products

is conserved among various strains of HIV, protection against subsequent challenge by a virulent strain of HIV that is homologous to, as well as strains heterologous to the strain from which the cloned gene is obtained, is enabled.

The i.m. injection of a DNA expression vector encoding gp160 results in the generation of significant protective immunity against subsequent viral challenge. In particular, gp160-specific antibodies and primary CTLs are produced. Immune responses directed against conserved proteins can be effective despite the antigenic shift and drift of the variable envelope proteins. Because each of the HIV gene products exhibit some degree of conservation, and because CTL are generated in response to intracellular expression and MHC processing, it is predictable that many virus genes give rise to responses analogous to that achieved for gp160. Therefore, the DNA vaccine formulations of the present invention offers a means to induce cross-strain protective immunity without the need for self-replicating agents.

The ease of producing and purifying DNA constructs compares favorably with traditional methods of protein purification, thus facilitating the generation of combination vaccines. Accordingly, multiple constructs, for example encoding gp160, gp120, gp41, or any other HIV gene may be prepared, mixed and co-administered. Because protein expression is maintained following DNA injection, the persistence of B- and T-cell memory may be enhanced, thereby engendering long-lived humoral and cell-mediated immunity.

It is also within the realm of the present invention to include additional components to the nucleic acid-adjuvant comprising vaccine formulations of the present invention. For example, HIV DNA-adjuvant-based formulations may also comprise antigenic protein as well as additional known adjuvants, such as saponin, to further enhance the immune response within the vertebrate host. It is within the purview of the skilled artisan to add such components to the vaccine formulations of the present invention.

It is also within the scope of the present invention to use DNA formulations which comprise DNA vaccine constructs providing an immune response to *M. tuberculosis*. A preferred antigen is the Ag85A, the Ag85B, or the Ag85C antigen. Vaccine constructs include but are not limited to (1) a construct which contains the either the mature Ag85A, B or C coding region fused with tPA signal sequence; (2) a construct which contains the mature Ag85A, B, or C coding region with no signal sequence; (3) a construct which contains Ag85A, B, or C with its own signal sequence.

The vaccine formulations of the present invention are exemplified utilizing a DNA plasmid encoding HA from the A/Georgia/93 strain. However, the skilled artisan will be directed to the use of additional influenza genes which encode antigens of interest. Such genes include but in not necessarily limited to human influenza virus nucleoprotein, basic polymerase 1, nonstructural protein1, hemagglutinin, matrix1, basic polymerase 2 of human influenza virus isolate A/PR/8/34, the nucleoprotein of human influenza virus isolate A/Beijing/353/89, the hemagglutinin gene of human influenza virus isolate A/Texas/36/91, or the hemagglutinin gene of human influenza virus isolate B/Panama/46/90.

It will also be known to the skilled artisan that the vaccine formulations of the present invention may comprise combinations of DNA plasmid constructs expressing HA from other clinical strains; including but not limited to, A/H1N1 (A/Texas/91), and B (B/Panama/90), as well as DNA constructs encoding the internal conserved influenza nucleoprotein (NP) and M1 (matrix) from both A (Beijing/89; H3N2) and B strains may be utilized in order to provide group-common protection against drifted and shifted antigens. The HA DNA will function by generating HA and resulting neutralizing antibodies against HA. This will be type-specific, with some increased breadth of protection against a drifted strain compared to the current licensed, protein-based vaccine. The NP and M1 constructs will result in the generation of CTL which will provide cross-strain protection with potentially lower viral loads and with acceleration of recovery from illness. The expected persistence of the DNA constructs (in an episomal, non-replicating, non-integrated form in the muscle cells) is expected to provide an increased duration of protection compared to the current vaccine.

The present invention relates to methods of generating an immune response in a vertebrate host, especially a human, wherein the vaccine formulations are administered to the host by any means known in the art of DNA vaccines, such as enteral and parenteral routes. These routes of delivery include but are not limited to intramusclar injection, intraperitoneal injection, intravenous injection, inhalation or intranasal delivery, oral delivery, sublingual administration, subcutaneous administration, transdermal administration, transcutaneous administration, percutaneous administration or any form of particle bombardment. The preferred methods of delivery are intramuscular injection, intranasal and oral based deliveries. An especially preferred method is intramuscular delivery. Regarding particle bombardment, use of aluminum adjuvants or calcium phosphate adjuvants as outlined

in this specification will improve the immune response produced by DNA delivered ballistically, on gold beads or as compacted particles. It will be well within the purview of the skilled artisan to deliver a formulation of the present invention as a simultaneous ballistic delivery of the DNA coated gold beads mixed with the aluminum or calcium adjuvant or as a subcutaneous or intramuscular injection of the adjuvant, followed by "gene gun" delivery of the DNA at or near the site of the adjuvant injection. The following examples are provided to further define the invention, without limiting the invention to the specifics of the examples.

EXAMPLE 1:
IN VITRO BINDING OF PLASMID DNA TO ALUMINUM ADJUVANTS

An experiment was designed to test the ability of various aluminum adjuvants to bind to plasmid DNA. Six different types of aluminum salts were examined, including aluminum hydroxide, aluminum hydroxyphosphate (precipitated in the presence of 3, 6, 12 or 24 mM sodium phosphate) and Adju-Phos®. The aluminum hydroxide (Alhydrogel®) and Adju-Phos® were purchased from Superfos Biosector, Denmark. The aluminum hydroxyphosphate adjuvants were prepared by preciptiating aluminum potassium sulfate in 3mM, 6mM, 12mM and 24mM sodium phosphate, respectively. The results of this binding study is summarized in Table 1. FR-9502 is a V1Jp based DNA plasmid vector with the gene encoding HA (A/Georgia/93). The FR-9502 plasmid DNA binds to all of the aluminum salts, except for Adju-Phos®. These results were based on a 15 minute, 16 hour or 72 hour incubation period using either 5 or 100 mcg/mL plasmid DNA and 450 mcg/mL of aluminum adjuvant, at 2-8 °C. For all the adjuvants except aluminum phosphate the binding studies were performed in saline because the presence of phosphate will change the surface charge of the adjuvant to become more like aluminum phosphate (Hem and White, 1995, Ch. 9, in *Vaccine Design: The Subunit and Adjuvant Approach, Eds.* Powell and Newman, Plenum Press (New York and London). The binding studies for aluminum phosphate were performed in PBS to allow a better comparison with the PBS control in the subsequent animal studies designed to examine the immune response. The samples were centrifuged and aliquots of the supernatant were taken and applied to a 1% agarose gel. Ethidium bromide staining of the gel following electrophoresis revealed the amount of total plasmid in solution by comparison to standards. It was also observed that there was no significant change

in the supercoiled content of plasmid DNA after incubation with Adju-Phos®. Therefore, no significant binding of plasmid DNA to aluminum phosphate-based adjuvant such as Adju-Phos® was observed, even after 3 days of incubation, based on quantitation of the supercoiled DNA bands in the gel. In contrast, partial binding in 15 minutes and complete binding of the DNA after 3 days was observed for Alhydrogel®) and various aluminum hydroxyphosphate adjuvants tested.

TABLE 1

Binding of plasmid DNA to aluminum adjuvants at 4 °C.

Type of Adjuvant[a]	[DNA][b]	Incubation	Results
Al(OH)3	5	16 hrs	complete binding
$^{0.5}$Al(OH)x(PO4)y	" "	" "	" "
^{1}Al(OH)x(PO4)y	" "	" "	" "
^{2}Al(OH)x(PO4)y	" "	" "	" "
^{4}Al(OH)x(PO4)y	" "	" "	" "
AlPO4	" "	" "	no binding observed
Al(OH)3	5 & 100	15 min	complete @ 5/partial @ 100
$^{0.5}$Al(OH)x(PO4)y	" "	" "	" "
^{1}Al(OH)x(PO4)y	" "	" "	" "
^{2}Al(OH)x(PO4)y	" "	" "	" "
^{4}Al(OH)x(PO4)y	" "	" "	" "
AlPO4	" "	" "	no binding observed
Al(OH)3	5 & 100	3 days	complete @ 5 and 100
$^{0.5}$Al(OH)x(PO4)y	" "	" "	" "
^{1}Al(OH)x(PO4)y	" "	" "	" "
^{2}Al(OH)x(PO4)y	" "	" "	" "
^{4}Al(OH)x(PO4)y	" "	" "	" "
AlPO4	" "	" "	no binding observed

[a]Type of adjuvant: 0.5 - 4 refer to aluminum hydroxyphosphate prepared by precipitation in 3, 6, 12 or 24 mM sodium phosphate, respectively. The points of zero charge for aluminum hydroxide, aluminum hydroxyphosphate, and aluminum phosphate are estimated to be ~ 11, 7 and 5). The aluminum concentration was 450 mcg/mL.

[b]DNA concentration is expressed as mcg/mL.

Plasmid DNA at 5 and 100 mcg/mL was incubated in the presence and absence of 450 mcg/mL Adju-Phos in PBS buffer for 10 days at 2-8 °C. Aliquots of the DNA were then subjected to agarose gel electrophoresis and ethidium bromide staining. Densitometry was used to scan a negative of a photograph of the gel to determine the binding state of the DNA and the amount of supercoiled, open-circular and linear forms, by comparison to DNA standards. The results indicated that the DNA in the 5 mcg/mL DNA samples with and without aluminum phosphate was 96% supercoiled, while the DNA in the 100 mcg/mL DNA samples was 95% supercoiled. Therefore, the presence of aluminum phosphate did not alter the stability of the DNA over this period of time. The gel lanes containing DNA from the 5 mcg/mL DNA samples with and without aluminum phosphate contained 15.0 and 15.1 ng of DNA, respectively. The gel lanes containing DNA from the 100 mcg/mL DNA samples with and without aluminum phosphate contained 14.9 and 13.7 ng of DNA, respectively. Therefore, there was no apparent binding of the DNA to the aluminum phosphate over the 10 day incubation period.

EXAMPLE 2
INHIBITION OF NUCLEASES IN MOUSE AND HUMAN SERA BY ALUMINUM PHOSPHATE.

This section examines the ability of aluminum phosphate to inhibit endogenous nucleases present in mouse and human sera. Since aluminum phosphate carries a negative surface charge one may reason that nucleases may bind to aluminum phosphate and lengthen the lifetime of the DNA *in vivo*, after intramuscular injection. The results indicate that the addition of 450 mcg/mL aluminum phosphate (Adju-Phos®) to a PBS solution containing 5 mcg/mL DNA and either 10% human serum or 2.5% mouse serum resulted in a significant inhibition of nuclease digestion of DNA. The results also suggest that in 10% bovine serum, different proteins were bound to the DNA in the presence of aluminum phosphate than in the absence of aluminum phosphate (as suggested by the change in mobility in a 1% agarose gel).

Example 3, *infra*, shows the effect of aluminum phosphate (Adju-Phos®) on the immune response in mice. To this end, these nuclease inhibition experiments were repeated. The experimental conditions were the same as described in the previous paragraph, except for an evaluation of doubling the aluminum

phosphate concentration to 900 mcg/mL. These data verify the previous results that aluminum phosphate inhibits nuclease activity in both human and mouse sera, and that increasing the aluminum phosphate concentration increases the degree of inhibition. It is also shown that lower nuclease activity was present in the supernatant of an aluminum phosphate (Adju-Phos®) / serum mixture. These data suggest that these nuclease proteins bind to aluminum phosphate in PBS, resulting in an inhibition of their activity, as evidenced by lower nuclease activity in the supernatant of an aluminum phosphate / serum mixture.

EXAMPLE 3

EFFECTS OF ALUMINUM SALTS ON HA DNA VACCINE POTENCY

In vivo potency studies in mice demonstrate that an aluminum phosphate formulation of DNA is substantially more potent (4- to 11-fold) than naked FR-9502 HA DNA in PBS, whereas HA DNA formulated with aluminum hydroxide or aluminum hydroxyphosphate resulted in lower responses than HA DNA in PBS (Table 2). This was true at 4 and 8 weeks after a single administration of the two doses of FR-9502 HA DNA tested; a limiting dose at which, based on numerous previous experiments, not all mice seroconvert (0.5 μg) and a moderate dose at which all mice seroconvert (10 μg). Importantly, this formulation appears to be both more potent at the lower dose and to have raised the ceiling on responses at the higher dose.

TABLE 2

Effect of Aluminum Adjuvants on HA DNA Potency in Mice

Hemagglutination Inhibition

Formulation	Dose (µg)	Wks	% seroconversion	GMT HI	SEM upper	SEM lower	P*	Fold Increase
PBS	0.5	4	60	14.4	5.5	4.0		
Al(OH)3	0.5	4	0	6.3	0.0	0.0		
1x AlHyd	0.5	4	0	6.3	0.0	0.0		
AlPO4	0.5	4	100	58.3	32.0	20.0	0.025	4.0
PBS	10	4	100	40.6	11.3	8.8		
Al(OH)3	10	4	30	10.9	3.9	2.9		
1x AlHyd	10	4	0	6.3	0.0	0.0		
AlPO4	10	4	100	303.1	126.1	89.1	0.000097	7.5
PBS	0.5	8	80	66.0	46.8	27.4		
Al(OH)3	0.5	8	0	6.3	0.0	0.0		
1x AlHyd	0.5	8	0	6.3	0.0	0.0		
AlPO4	0.5	8	100	459.5	163.4	120.5	0.0038	7.0
PBS	10	8	100	81.2	27.2	20.4		
Al(OH)3	10	8	60	37.9	28.3	16.2		
1x AlHyd	10	8	0	6.3	0.0	0.0		
AlPO4	10	8	100	800.0	348.1	242.5	0.000059	9.9

IgG ELISA

Formulation	Dose (µg)	Wks	GMT ELISA	SEM upper	SEM lower	P*	Fold Increase
PBS	0.5	8	12800	10211	5680		
AlPO4	0.5	8	144815	138639	70830	0.011	11.3
PBS	10	8	25600	15983	9839		
AlPO4	10	8	258031	136439	89248	0.0017	10.1

*two-sided t-test for independent samples

Female BALB/c mice (10/group) were inoculated with FR-9502 HA DNA (A/Georgia/93) at doses of 0.5 or 10 μg and antibody titers (HI and IgG ELISA) were determined at 4 and 8 weeks after a single administration.

Analysis of the immunoglobulin isotypes reveals that the enhancing effects of aluminum phosphate (Adju-Phos®) do not result in qualitative differences in the types of antibody produced by HA DNA (Table 3). Aluminum adjuvants tend to induce a strong Th2-type of helper T cell response against co-injected protein which is often accompanied by a predominance of IgG1 antibodies in mice.

TABLE 3

Immunoglobulin Isotype Analysis

			GMT ELISA				
Formulation	Dose (μg)	Wks	IgG1	IgG2a	IgG2b	IgG3	IgG2a:IgG1
PBS	0.5	8	2,786	9,700	696	303	3.48
AlPO4	0.5	8	9,051	86,107	21,526	1,131	9.51
PBS	10	8	3,200	25,600	2,262	336	8.00
AlPO4	10	8	29,863	221,244	34,836	1,600	7.41

Sera taken from mice (10/group) 8 weeks after inoculation of DNA with and without aluminum phosphate were analyzed for immunoglobulin isotypes by an ELISA.

Additional studies disclosed in Example Section 7 confirm that co-administration of aluminum phosphate with plasmid DNA encoding influenza HA enhanced the magnitude and duration of anti-HA antibodies in mice, compared to that induced by naked HA DNA alone. At 4, 8 and 17 weeks after a single inoculation, antibody titers, as measured by the functional assay hemagglutination inhibition (HI), were higher in mice vaccinated with the aluminum phosphate formulation of HA DNA. A wide range of aluminum phosphate and DNA doses are confirmed to be effective in mice, whether measured by HI or an ELISA. The enhancing effects of aluminum phosphate on a DNA construct encoding a second influenza antigen (nucleoprotein or NP) was also tested in mice and the data is also disclosed in Example Section 7. As before, antibody responses were enhanced 5- to 50-fold by formulation of DNA with aluminum phosphate. In addition, it is shown that cytotoxic T lymphocyte responses against NP in these mice were not detrimentally affected.

EXAMPLE 4
EFFECT OF ALUMINUM PHOSPHATE (Adju-Phos®) ON IN VIVO GENE EXPRESSION

An experiment to test the effect of (Adju-Phos®) on *in vivo* gene expression was conducted. A plasmid encoding secreted alkaline phosphatase (SEAP) previously shown to express in non-human primates was used. This experiment compared the level of SEAP in the serum 3 days after intramuscular injection of either 1 mcg or 10 mcg of SEAP plasmid DNA into mice, formulated in either PBS or PBS containing 450 mcg/mL aluminum phosphate. Ten mice were used in each group. The results suggest that the presence of aluminum phosphate did not have a significant effect on SEAP levels in the serum, 3 days post-injection. These results suggest that the increase in immune response obtained with aluminum phosphate may not have been the result of an overall increase in gene expression.

Example Sections 1-4 show that a DNA vaccine formulation comprising an aluminum-phosphate-based adjuvant and HA plasmid DNA (A/Georgia/93) in PBS substantially increased the humoral immune response to the expressed HA protein in mice (approximately 4- to 11-fold enhancement in antibody titer). In contrast, HA DNA formulated with aluminum hydroxide or aluminum hydroxyphosphate adjuvants shown to bind DNA inhibited the immune response to HA protein (compared to plasmid DNA alone in PBS). *In vitro* binding studies of plasmid DNA to different types of aluminum adjuvants demonstrated that plasmid DNA does not bind to the negatively charged aluminum phosphate (in PBS or in 0.9% saline). However, plasmid DNA does bind to the more positively charged aluminum hydroxide and more positively charged aluminum hydroxyphosphate adjuvants in saline. Therefore, aluminum phosphate-based adjuvants tending to posses a negative surface charge are effective non-binding adjuvants for DNA vaccine formulations.

EXAMPLE 5
IN VITRO BINDING OF PLASMID DNA TO ALUMINUM HYDROXYPHOSPHATE ADJUVANTS

Four solutions were prepared as shown below in Table 4 to determine if plasmid DNA binding to aluminum hydroxyphosphate could be prevented by the addition of phosphate buffer. Each solution contained plasmid DNA at 100 mcg/mL and aluminum hydroxyphosphate.

Table 4

Solution	Formulation
1	DNA in 0.9% NaCl
2	DNA in saline containing 450 mcg/mL Al
3	DNA in PBS (6 mM phosphate, 150 mM NaCl) with 450 mcg/mL Al
4	DNA in PBS (12 mM phosphate, 150 mM NaCl) with 450 mcg/mL Al

The solutions were prepared, mixed by inversion and incubated at 4°C. After 15 minutes of incubation, the solutions were centrifuged in a microcentrifuge for 2 minutes to pellet the adjuvant. Aliquots of the supernatant were taken, diluted 20-fold with PBS and subjected to a UV absorbance scan from 400 to 220 nm. The DNA concentration in the supernatant was determined, based on the assumption that an absorbance of 1.0 at 260 nm is produced by DNA at 50 mcg/mL. The results are shown below in Table 5.

Table 5

Solution	[DNA] in supernatant after 15 minutes
1	96.7 mcg/mL
2	11.8 mcg/mL
3	100.0 mcg/mL
4	99.4 mcg/mL

The results indicate that most of the plasmid DNA bound to aluminum hydroxyphosphate within 15 minutes in 0.9% saline, but did not bind to the adjuvant in PBS. Aliquots of the supernatants were also analyzed by agarose gel electrophoresis after 15 minutes and 5 days of incubation at 4°C. The results indicated that there was no detectable DNA in the supernatant of solution 2 after 15 minutes or after 5 days of incubation. However, a comparison of the amount of DNA in the supernatants from solutions 1, 3 and 4 indicated that there was no significant binding of the DNA to the aluminum adjuvant in either 6 or 12 mM phosphate, over 5 days at 4°C. Therefore, it will be within the purview of the skilled artisan to utilize an adjuvant in a DNA vaccine formulation that may, in some formulations, substantially bind DNA. This adjuvant may be useful by including a phosphate buffer or other buffer that results in an inability to substantially bind DNA within this DNA vaccine formulation.

The ability of aluminum hydroxyphosphate to enhance the immune response generated by plasmid DNA containing the HA-Georgia Influenza gene has been examined in two experiments, formulated in both saline and PBS. The results (Table 6) indicate that aluminum hydroxyphospate did not enhance the immune response (based on geometric mean titers to the HA protein antigen) to the Influenza DNA vaccine if it was formulated in saline, but it did enhance the immune response if formulated in PBS. Agarose gel electrophoresis of the supernatants of these formulations indicated that the DNA was completely bound to the aluminum hydroxyphosphate in the saline formulation, but was not bound in the PBS formulation. These results show that the DNA must be in solution and not bound to the aluminum adjuvant in order to enhance the immune response to a DNA vaccine.

TABLE 6

Enhancement of immune responses to an Influenza DNA vaccine in mice by aluminum adjuvants.

Experiment	Formulation	4 week GMT[a]	seroconverters	8 week GMT[a]	seroconverters
I-79	10 mcg DNA in PBS	70.7	10/10	132	10/10
I-79	10 mcg DNA 45 mcg AlhydroxP in saline	6.3	0/10	6	10/10
I-79	10 mcg DNA 45 mcg AlPO4 in PBS	459.5	10/10	1132	10/10
I-99	10 mcg DNA in PBS	25	8/10	12,800[b]	10/10
I-99	10 mcg DNA 45 mcg alhydroxP in PBS	107	9/10	51,200[b]	10/10
I-99	10 mcg DNA 45 mcg AlPO4 in PBS	229	10/10	33,779	10/10

[a] refers to the geometric mean titer to the HA protein antigen.

[b] 8-week GMT was determined by ELISA assay

EXAMPLE 6
EFFECT OF ALUMINUM PHOSPHATE ON POTENCY OF *INFLUENZA* DNA VACCINES

1. *Influenza HA DNA Vaccine* - Female BALB/c mice (10/group) were inoculated with FR-9502 HA DNA (A/Georgia/93) at doses of 0.5 μg or 10 μg and antibody titers (HI and IgG ELISA) were determined at 4 and 8 weeks after a single administration. Controls included inoculation with 0.5 or 10 μg of HA DNA (A/Georgia/93) in PBS. Unless indicated otherwise, $AlPO_4$ was co-administered at 450 μg/ml along with HA DNA. HA DNA potency in Figure 1A and 1B is reported as the production of neutralizing antibodies as measured *in vitro* by a hemagglutinin inhibition (HI) assay. These data show that at 4 weeks (Figure 1A) and 8 weeks (Figure 1B) post-injection, a significant enhancement of HA DNA vaccine potency is measured when utilizing a DNA vaccine formulation comprising 450 μg /ml $AlPO_4$, with DNA at doses of both 0.5 μg and 10 μg. Table 7 shows a similar enhancement by adding an aluminum phosphate adjuvant as measured by HA ELISA.

TABLE 7

Generation of Humoral Response in Mice

DNA	Adjuvant	Dose (μg)	ELISA (GMT)
HA(A/Georgia/93)	PBS (None)	0.5	12,800
HA(A/Georgia/93)	AlPO4	0.5	144,820
HA(A/Georgia/93)	PBS (None)	10	25,600
HA(A/Georgia/93)	AlPO4	10	258,030

A HA DNA vaccine formulation comprising aluminum phosphate as an adjuvant did not significantly alter the IgG antibody profile. As noted *supra*, Table 3 shows that PBS- and AlPO4- based DNA vaccine formulations (measured at 0.5 and 10 μg doses at 4 and 8 weeks post-injection) result in similar isotype profiles of IgG1, IgG2a, IgG2b and IgG3 in response to HA DNA vaccination. In addition the profile of the humoral response to HA DNA vaccination, the duration of the response in mice also indicates that the rise and fall of HA neutralizing antibodies follows a similar path, regardless of whether the formulation contained PBS or AlPO4. Data in Figure

2A (0.5 μg HA DNA) and Figure 2B (10 μg HA DNA) show induction of HA neutralizing antibodies at 4, 8 and 17 weeks post-infection. In both PBS- and AlPO4-based DNA vaccine formulations, a drop in HA antibodies is seen from 8 weeks post-injection to 17 weeks post-injection.

Additional experiments show that the optimal effect of AlPO4 as an adjuvant to DNA vaccination procedures occurs when the DNA and AlPO4 are co-administered to the host. Table 8 compares the ability of HA DNA to elicit neutralizing antibodies when AlPO4 is either co-injected with the DNA or administered to mice three days prior to 3 days after DNA immunization.

TABLE 8

Effect of Co-Administration of AlPO4/HA DNA on Enhancement of HI Titer in Mice

DNA (10mcg)	$AlPO_4$ / DNA Admin.[1]	HI Titer (GMT)[2]
HA(A/Georgia/93)	none (PBS)	25
HA(A/Georgia/93)	AlPO4 -co-injected	229
HA(A/Georgia/93)	AlPO4 - 3 d prior	66
HA(A/Georgia/93)	AlPO4 - 3 d after	35

[1] $AlPO_4$ at 450 mcg/ml.

[2] At 4 weeks post-injection.

Similar results were recorded when HA antibody production was measured by an HA ELISA assay. These data show that the optimal time of administering AlPO4 as a DNA vaccine adjuvant is at or substantially near the time that the DNA vaccine is administered. Therefore, the DNA/AlPO4 formulations of the present invention provide a preferred formulation for stimulating an *in vivo* humoral response following DNA vaccination.

Additional experiments show that AlPO4 acts as an adjuvant over a wide range of concentrations which may be envisioned by the skilled artisan. Figure 3A and Figure 3B show that various AlPO4 concentrations co-administered within various dose ranges of HA DNA promote an enhanced humoral response at least 4 weeks post-injection. It is evident from these results that a wide AlPO4 dose range will be effective in providing the DNA adjuvant effect disclosed and exemplified

within this specification. Therefore, the data presented in this Example Section show that AlPO4 acts as a adjuvant to significantly increase humoral responses upon DNA vaccination. This increased humoral response is not dependent upon specific dose combinations of adjuvant and DNA. Instead, higher DNA doses tend to result in somewhat more pronounced antibody production up to about a dose of 10 μg DNA in mice, whereas the adjuvant effect of AlPO4 remains steady over a large dose range. This data serves as an effective guidepost to the skilled artisan in determining DNA and adjuvant dose ranges for the host of interest, including but not limited to human and/or veterinary applications.

2. *Influenza NP DNA Vaccine* - Female BALB/c mice (10/group) were inoculated with a DNA plasmid encoding nucleoprotein (NP) from influenza virus A/PR/8/34 (H1N1) at doses of 0.5 μg or 50 μg and anti-NP titers were determined at 6 weeks after a single injection and at 3 weeks post two injections. Unless indicated otherwise, AlPO4 was co-administered at 450 μg/ml along with NP DNA. NP DNA potency is reported in Figure 4 as anti-NP antibodies measured as the geometric mean ELISA titer. Serum samples were collected from groups of 3 mice at the time of sacrifice for cellular immune responses. These data show that anti-NP antibody production in response to innoculation with a NP DNA plasmid construct is increased when utilizing a DNA vaccine formulation comprising 450 μg/ml AlPO4, with DNA at doses of both 0.5 μg and 50 μg.

Figure 5A (IL-2), Figure 5B (INF-γ), Figure 5C (IL-4) and Figure 5D (IL-10) show that innoculation of mice with a NP DNA plasmid/AlPO4 vaccine formulation provided no significant alteration of cytokine secretion as compared to a NP DNA plasmid/PBS formulation injected at identical doses, as measured from spleen cells pooled from 3 mice/group.

In order to show the extent of a cellular response to innoculation with a NP DNA plasmid construct, with or without the addition of AlPO4, cytotoxic T lymphocytes were generated from mice that had been immunized with DNA or that had recovered from infection with A/PR/8/34. Control cultures were derived from mice that had been injected with control DNA and from uninjected mice. Single cell suspensions were prepared from pools of 3 spleens/group, red blood cells were removed by lysis with ammonium chloride, and spleen cells were cultured in RPMI 1640 supplemented with 10% Fetal Bovine Serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, 0.01 M HEPES (pH 7.5), and 2 mM l-glutamine. An equal number of autologous, irradiated stimulator cells, pulsed for 60 minutes with the H-

.2K^d-restricted peptide epitope NP147-155 (Thr-Tyr-Gln-Arg-Thr-Arg-Ala-Leu-Val, SEQ ID NO: 13) at 10 μM or infected with influenza strain A/Victoria/73, and 10 U/ml recombinant human IL-2 (Cellular Products, Buffalo, NY) were added and cultures were maintained for 7 days at 37°C with 5% CO2 and 100% relative humidity. The cytotoxicity assays were performed as described by Ulmer et al. (1993, *Science* 259:1745-1749). Target cells labeled with Na^{51}CrO were pulsed with synthetic peptide NP147-155 at a concentration of 10 μM. The target cells were then mixed with CTL at designated effector:targer cell ratios in 96-well plates, and incubated at 37°C for four hours in the presence of 5% CO2. A 20 μl sample of supernatant from each cell mixture was counted to determine the amount of ^{51}Cr released from target cells and counted in a Betaplate scintillation counter (LKB-Wallac, Turku, Finland). Maximal counts, released by addition of 6M HCl, and spontaneous counts released without CTL were determined for each target preparation. Percent specific lysis was calculated as: [(E -S)/(M -S] x 100, where E represents the average cpm released from target cells in the presence of effector cells, S is the spontaneous cpm released in the presence of media only, and M is the maximum cpm released in the presence of 2% Triton X-100. The results in Figure 6A, Figure 6B, Figure 6C and Figure 6D show a minimal effect of the presence of AlPO4 on induction of an CTL response by innoculation with a NP DNA plasmid construct. In these studies BALB/c mice were injected in the quadriceps of both legs with plasmid DNA encoding A/PR/8/34 (H1N1) with either 5 μg or 50μg of plasmid DNA, in PBS or AlPO4. The level of % specific lysis was determined through lymphocyte cultures derived from mice 6 weeks post injection. The results show that the CTL response was similar at both doses for both peptide-pulsed cells and flu-infected cells. Similar results were obtained for 5 μg or 50 μg doses at 3 weeks post 2 injections.

EXAMPLE 7
EFFECT OF ALUMINUM PHOSPHATE ON POTENCY OF HEPATITIS B DNA VACCINES

DNA Constructions - The major envelope protein (HBs) from hepatitis B virus was subcloned into expression vectors derived from V1J, derived from a pUC19 plasmid containing the human cytomegalovirus (CMV) immediate early

promoter with its intron A sequence, multiple restriction sites (including Bgl II) for cloning and the bovine growth hormone polyadenylation signal sequence. The HB DNA plasmid vector expressing the *adw* subtype is V1Jns.S. The HB DNA plasmid vector expressing the *ayw* subtype is V1R.S, which was prepared by subcloning the S gene from a pBR322 plasmid that contained the entire HBV genome into the BglII restriction site of the V1R. Expression of the S gene was confirmed in RD cells (a human myoblast cell line) by calcium phosphate-mediated transfection using the CellPhect kit (Pharmacia) and detection of the HBsAg using the Auzyme EIA kit (Abbott Labs).

Anti-HBs EIA (total antibody) - A microtiter plate modification of the AUSAB EIA kit (Abbott Labs, N. Chicago, IL) was used to quantify antibodies to hepatitis B surface antigen (HBsAg). Costar EIA 96-well flat bottom plates (Costar, Cambridge MA, #3591) were coated overnight at 4°C with recombinant HBsAg (prepared *e.g.*, U.S.Patent Nos. 4,769,238; 4,935,235; and 5,196,194) at 4 µg/ml in Tris-saline, pH 9.5. Plates were washed 3 times with PBS and then blocked with 175 µl/well of PBS/5% FCS/ 0.1% azide for 2 hours at room temperature or overnight at 4°C. Five-fold serial dilutions were made (in duplicate) in 8 consecutive wells of the plate for each serum sample. The plates were then incubated overnight at 4°C. After 3 wash cycles with PBS (using a TiterTech plate washer [ICN, Huntsville, AL]), a developing reagent (Abbott AUSAB EIA kit) consisting of equal volumes of biotin-conjugated HBsAg and an anti-biotin-enzyme conjugate was added to each well of the plate. After 4 hours at room temperature, the plates were washed 6 times and then 100 µl per well of OPD substrate (Abbott) was added to each well. The reaction was stopped after 30 minutes with the addition of 50 µl per well of 1 N H_2SO_4. Optical densities were read at 490 nm and 650 nm using a Molecular Devices microplate reader (Molecular Devices, Menlo Park, CA). Anti-HBs titers (in mIU/mL) were calculated by the Softmax computer program (version 2.32) using a standard curve generated using a 4-parameter fit algorithm. Since the assay is species-independent, a set of human serum standards (Abbott quantitation kit) was used to generate the standard curve so that titers could be quantified relative to a reference standard in mIU/mL.

Anti-HBs EIA (isotype-specific) - Microtiter plates were coated with HBsAg and blocked as described above. Five-fold serial dilutions were made (in duplicate) in 8 consecutive wells of the plate for each serum sample. The plates were then incubated overnight at 4°C. After 3 wash cycles with PBS (using a TiterTech

plate washer), alkaline phosphatase-conjugated goat anti-mouse immunoglobulin reagents specific for mouse IgG1 or mouse IgG2a isotypes (Southern Biotechnology Associates, Birmingham, AL) were added at a final dilution of 1:2000. After 2 hours at 37°C, the plates were washed 6 times using a TiterTech plate washer, and then 60 µl per well of the enzyme substrate (p-nitrophenylphosphate [Sigma Chemical Co., St. Louis, MO] dissolved at 1 mg/mL in Tris saline, pH 9.5) was added. After 30 minutes at room temperature, the reaction was stopped with the addition of 60 µl/well of 3N NaOH. Optical densities were read at 405 nm using a Molecular Devices microplate reader. Data were collected using the Softmax computer program. A standard curve was generated using mouse monoclonal anti-HBs antibodies of the IgG1 (catalogue # 16021, Pharmingen, San Diego, CA) or IgG2a (cat. # 16011D, Pharmingen) isotypes.

Antibody concentrations relative to each isotype standard were calculated as described previously (Caulfield and Shaffer, 1984, *J. Immunol. Methods* 74: 205-215). Briefly, to calculate titers, an OD value of 0.1 units was set as the endpoint. The log 5 titer (t) is determined by interpolation using the following formula:

$$t = x - ((0.1 - L)/(H - L)$$

where L = OD value of the first log 5 dilution giving an OD value below 0.1; H = OD value of the log 5 dilution closest to, but above the cutoff (θ.1); x = the well number that has the OD value L.

The antibody concentration (c) in experimental samples is determined by comparing the endpoint titer in experimental wells with that of the standard curve by the following formula:

$$c = A \times 5^{(t - s)}$$

where A = the antibody concentration of the standard; s = the log 5 titer of the standard; t = the log 5 titer of the unknown. For example, if the log 5 endpoint titer of the standard (100 ng/ml) is 2.6 and the value of the unknown is 3.4, the concentration of antibody in the unknown would be:

$$c = 100 \times 5^{(3.4 - 2.6)} = 362 \text{ ng/ml}.$$

Cytotoxic T Lymphocyte Assays (CTL assays) - The CTL assays were performed as reported in Ulmer et al. (1993, *Science* 259: 1745-1749), and essentially as described in Example Section 6. Briefly, BALB/c mice were injected twice with a vaccine formulation consisting of HBV DNA plus aluminum phosphate or with naked HBV DNA. A single cell suspension of effector cells was then prepared and cultured

in vitro with HBs peptide (28-39)-pulsed syngeneic stimulator cells. The cell suspension was assayed 7 days later for CTL activity against ^{51}Cr-labeled P815 cells.

The syngeneic stimulator cells were prepared as a single cell suspension from the spleens of unimmunized BALB/c mice as follows. After lysis of red blood cells with ammonium chloride buffer (Gibco BRL ACK buffer), the cells were washed by centrifugation for 10 minutes at 1200 rpm (Jouan centrifuge model CR422), resuspended in DMEM culture medium (Gibco BRL #11965-092), and then irradiated using a ^{60}Co source to deliver 2,000 - 4,000 rads. The cells were then pulsed with a 10 μM final concentration of the H-2 K^d peptide HBs (28-39) (Chiron Mimetopes, Clayton, Victoria, Australia) which has the sequence Ile-Pro-Gln-Ser-Leu-Asp-Ser-Trp-Trp-Try-Ser-Leu [SEQ ID NO:14] (Schirmbeck et al., 1994, *J. Virol.* 68: 1418-1425). The cells were mixed approximately every 20 minutes for 1.5 - 2.5 hours and then washed 3 times with RPMI-1640 medium. Effector cells were prepared as single cell suspensions from spleens of immunized mice as described and then co-cultured with an approximately equal number of peptide-pulsed stimulator cells for 7 days at 37° C (5% CO_2) in "K" medium.

P815 (H-2^d) mouse mastocytoma cells (ATCC, Rockville, MD) were radiolabeled by overnight culture with 0.5 - 1.2 mCi ^{51}Cr (Amersham, cat. # CJS.4) added to 75 cm^2 culture flasks (Costar #3376) containing ~ 5 x 10^5 cells per mL in a volume of 10 mL. The labeled cells were centrifuged at 1200 rpm for 5 minutes and the supernatant removed by aspiration. The cells were washed, counted, resuspended in DMEM culture medium at ~10^6 cells per mL and then pulsed with 10 μM HBs (28-39) peptide at 37° C for 2-3 hr with frequent mixing. The target cells were then washed and adjusted to 10^5 cells per mL for plating. Meanwhile, effector cells from the 7 day restimulation cultures were harvested, washed, and added to triplicate wells of V bottom microtiter plates (Costar #3898) at 60 x 10^5, 30 x 10^5, 15 x 10^5, and 7.5 x 10^5 cells per mL. The ^{51}Cr-labeled target cells were plated at 10^4 cells per well in 100 μl "K" medium to achieve effector:target ratios of 60:1, 30:1, 15:1, and 7.5:1. Triplicate wells containing only target cells cultured in 0.2 mL of medium served as controls for spontaneous ^{51}Cr release whereas triplicate wells containing target cells cultured in 0.2 mL of medium containing 1.0 % Triton X-100 detergent (Sigma #T6878) served as controls for maximum ^{51}Cr release. The plates were incubated for 4 hours at 37°C in a 5% CO_2 incubator and then centrifuged at 1200 rpm for 5 minutes to pellet the remaining target cells. The supernatants (20 μl) were then harvested using an Impact multichannel pipetor (Matrix Technology, Lowell MA,

model #6622) and then transferred to Betaplate filter mats (Wallac #1205-402). The mats were dried and then transferred to plastic bags which were sealed after the addition of ~11 mL of scintillation fluid. A Betaplate model 1205 scintillation counter (Wallac) was used to quantify the radioactive ^{51}Cr contained in each spot on the mat corresponding to each well of the original 96-well plate. The % specific lysis was determined as set forth in Example Section 7.

Adjuvant effect of aluminum phosphate for V1R.S - A study comparing anti-HBs antibody production in mice inoculated with (1) a commercial hepatitis B vaccine (Recombivax HB®); (2) purified hepatitis B surface antigen without an adjuvant, and (3) V1R.S with aluminum phosphate and (4) V1R.S without aluminum phosphate was performed. Animals were utilized as described in Example Section 6. Female BALB/c mice were inoculated with the plasmid DNA construct V1R.S at a 100 μg dose either in the presence of 450 μg /ml aluminum phosphate or in the absence of the adjuvant. As controls, one microgram of Recombivax HB® and 1 μg of HBsAg were injected into mice and bleeds were taken 21, 42 and 63 days after inoculation. Anti-HBs antibody production is shown in Figure 7. The antibody response to a HBV DNA vaccine (which encodes the surface antigen from hepatitis B virus) was enhanced approximately 100-fold by formulation with aluminum phosphate. The adjuvanted DNA vaccine generates a response equivalent to that induced with Recombivax HB®.

HB DNA Dosage Rates in the Presence of AlPO4 - V1R.S DNA was formulated at three dose levels (1.0, 10, and 100 μg) with a constant (450 μg/ml) concentration of aluminum adjuvants (aluminum phosphate and aluminum hydroxyphosphate) and then tested for the ability to induce anti-HBs antibodies in mice. Figure 8 shows that 6 weeks after a single injection of vaccine, the response to a 10 μg dose of HBV DNA vaccine formulated with aluminum phosphate was superior to that induced with 100 μg of the naked DNA vaccine. Figure 9 shows that injection of mice at day 0 and day 42 with DNA formulated at three dose levels (1.0, 10, and 100 μg) with a constant (450 μg/mL) concentration of aluminum adjuvants. Anti-HBs antibodies in BALB/c mice were tested three weeks later at day 63 of the experiment. By comparison with the data shown in Figure 7, boosting with a second dose of DNA vaccine formulated with aluminum phosphate generated a > 10-fold rise in anti-HBs titers. Consistent with a single dosing as shown in Figure 7, the response to a 10 μg dose of HBV DNA vaccine formulated with aluminum phosphate was superior to that induced with 100 μg of the naked DNA vaccine. Formulation of

DNA in saline with aluminum hydroxide or aluminum hydroxyphosphate adjuvants was advantageous only at the 100 μg dose of DNA under conditions in which the aluminum adjuvants are saturated and free DNA is present. At lower doses of DNA where it is known that the DNA binds completely to aluminum hydroxide or aluminum hydroxyphosphate, the response is lower than that obtained with equivalent doses of naked DNA.

HBV DNA/AlPO4 Induction of CTL Response - After two injections of the HBV DNA vaccine plus aluminum phosphate adjuvant, spleen cells from BALB/c mice were restimulated *in vitro* with HBs peptide (28-39) and then assayed 7 days later for CTL activity against ^{51}Cr-labeled P815 cells. Figure 10 shows that the formulation of the HBV DNA vaccine with or without aluminum phosphate generated equivalent CTL responses. There was no lysis of control P815 cells not pulsed with the HBs peptide indicating that lysis of the HBs peptide-pulsed cells was the result of activation of specific CTLs rather than natural killer (NK) cells that would be expected to lyse target cells indiscriminately. Therefore, a major advantages of naked DNA vaccination (i.e., induction of CTL responses) is preserved when the DNA is formulated with aluminum phosphate.

EXAMPLE 8

ADJUVANT EFFECT OF ALUMINUM PHOSPHATE FOR HBs DNA VACCINE TESTED IN LOW RESPONDER MICE

A significant proportion of humans are non-responders to a standard 3-dose regimen of the current hepatitis B vaccines (Alper, et al., 1989, *J. Eng. J. Med.* 321:708-712). This problem was addressed using the aluminum phosphate adjuvant in a preclinical animal model. Low responder (C3H) or high responder (BALB/c) mice were immunized with two doses of 1.0, 10, or 100 μg of HBs DNA vaccine formulated with or without aluminum phosphate. As shown in Table 9, formulation of the DNA vaccine with aluminum phosphate enables the generation of an anti-HBs antibody response in both high responder (BALB/c) and low responder (C3H) mice given the 100 μg dose of DNA that is equivalent to the response to a 1 μg dose of a conventional HBs protein vaccine. It is of note that in the absence of the aluminum adjuvant, the response to the DNA vaccine was only 6.3 mIU/mL which is just above the detectable limit of ~1.0 mIU per mL. Thus, the aluminum phosphate adjuvant combines the desired attributes of protein-based vaccines (i.e. the induction of high

antibody titers) with the ability of DNA vaccines to induce cell-mediated antibody responses (see Example Section 7).

TABLE 9

Anti-HBs response of high vs. low responder mice to HBsDNA ± AlPO4

			Anti-HBs GMT (mIU/mL)	
Immunogen	Dose	Adjuvant	BALB/c	C3H
HBsAg	1 µg	Al(OH)PO4	8,045.0	91.4
HBs DNA*	100 µg	none	415.0	6.3
	10 µg		19.8	1.7
	1 µg		3.3	1.3
	100 µg	AlPO4	4,408.0	111.5
	10 µg		280.0	23.7
	1 µg		6.8	4.8

*V1R.S (ayw) (XLpl.11) 5286-115 V44

EXAMPLE 9

EFFECT OF ALUMINUM PHOSPHATE ON POTENCY OF HIV DNA VACCINES

DNA plasmid V1Jns/tPA/opt gag was constructed from the vector V1Jns, described in WO 97/3115 and herein incorporated by reference. The optimized gag sequence within V1Jns was constructed as follows: In order to provide an heterologous leader peptide sequence to secreted and/or membrane proteins, V1Jn was modified to include the human tissue-specific plasminogen activator (tPA) leader. Two synthetic complementary oligomers were annealed and then ligated into V1Jn which had been BglII digested. These oligomers have overhanging bases compatible for ligation to BglII-cleaved sequences. After ligation the upstream BglII site is destroyed while the downstream BglII is retained for subsequent ligations. Both the junction sites as well as the entire tPA leader sequence were verified by DNA sequencing. Additionally, in order to conform with our consensus optimized vector V1Jns (=V1Jneo with an SfiI site), an SfiI restriction site was placed at the

KpnI site within the BGH terminator region of V1Jn-tPA by blunting the KpnI site with T4 DNA polymerase followed by ligation with an SfiI linker (catalogue #1138, New England Biolabs). This modification was verified by restriction digestion and agarose gel electrophoresis.

Gene segments were converted to sequences having identical translated sequences but with alternative codon usage as defined by Lathe (1985, *J. Mol. Biol.* 183: 1-12), and described in WO 97/3115. The methodology described below to increase of expression of HIV *gag* gene segments was based on the hypothesis that the known inability to express this gene efficiently in mammalian cells is a consequence of the overall transcript composition. Thus, using alternative codons encoding the same protein sequence may remove the constraints on expression of *gag*. The specific codon replacement method employed may be described as follows:

1. Identify placement of codons for proper open reading frame.
2. Compare wild type codon for observed frequency of use by human genes.
3. If codon is not the most commonly employed, replace it with an optimal codon for high expression in human cells.
4. Repeat this procedure until the entire gene segment has been replaced.
5. Inspect new gene sequence for undesired sequences generated by these codon replacements (e.g., "ATTTA" sequences, inadvertent creation of intron splice recognition sites, unwanted restriction enzyme sites, etc.) and substitute codons that eliminate these sequences.
6. Assemble synthetic gene segments and test for improved expression.

These methods were used to create the following synthetic gene segments for HIV *gag* creating a gene comprised entirely of optimal codon usage for expression. An artisan of ordinary skill in the art will understand that similar vaccine efficacy or increased expression of genes may be achieved by minor variations is the procedure or by minor variations in the sequence.

DNA plasmid V1Jns/tPA/gp140 optA was constructed as described above for optimization and specifically as described in PCT International Application No. WO 97/31115.

Female Balb/C mice (10/group) were inoculated with V1Jns/tPA/gp140optA and V1Jns/tPAopt gag at doses of 10 μg (5 μg of each construct) or 100 μg (50 μg of each construct). Aluminum phosphate (AlPO4 from a 2% solution), or CaPO4 (27.5mg/100ml stock) was added at final amounts of 11 μg for AlPO4, and 19 μg for CaPO4. Controls included inoculations formulations with adjuvant and/or no DNA or DNA with no adjuvant.

Figure 11 shows the effects of various adjuvants with a HIV eng/gag DNA vaccine formulation on gp120 and gag antibody responses in inoculated mice. Antibody production was measured by ELISA. As shown in Example 7 with HBV DNA vaccines, CTL responses with and without AlPO4 were approximately equal. Therefore, use of an adjuvanted HIV env/gag formulation did not decrease the ability of the vaccine to promote a specific CTL response.

EXAMPLE 10

DOSE-RESPONSE RELATIONSHIP OF CALCIUM PHOSPHATE AS AN ADJUVANT FOR A DNA VACCINE.

Calcium phosphate (at different concentrations) was compared with a standard concentration of aluminum phosphate as an adjuvant for HBV DNA vaccine. Three dose levels (10, 100, and 1000 μg/mL) of the HBV DNA vaccine were formulated to contain 10, 3.3, 1.0, or 0.3 mg/mL calcium phosphate or 0.45 mg/mL aluminum phosphate. A total of 0.1 mL of formulated vaccines was injected into two intramuscluar sites of BALB/c mice to achieve DNA vaccine dosages of 1.0, 10, or 100 μg. As shown in Table 10, HBV DNA formulated with the three lower concentrations of calcium phosphate increased the anti-HBs response to the 100 μg vaccine dose by approximately 10-fold. At the highest concentration of calcium phosphate, only 7% of the DNA remained unbound to the adjuvant, and the response to this formulation was increased by less than 2-fold. In the cohorts of mice receiving the 10 μg dose of HBV DNA, the aluminum phosphate had a powerful adjuvant effect, increasing the response ~40-fold compared with the group receiving 10 μg of naked DNA. By contrast, DNA formulated with calcium phosphate at 10, 3.3, or 1 mg per mL induced a response that was 10-fold lower than that to naked DNA. Only in the group receiving the DNA vaccine formulated with the lowest concentration of calcium phosphate (0.3 mg/mL) was an adjuvant effect observed for the 10 μg dose level of the DNA vaccine. An analysis of separately prepared vaccines (10 μg DNA dose) containing calcium phosphate indicated that the percent unbound DNA was

73%, 26%, 1%, and 0% in vaccines containing 0.3, 1.0, 3.3, and 10 mg/mL calcium phosphate, respectively. Taken together, these results indicate that calcium phosphate can be an effective adjuvant for a DNA vaccine only if the formulation contains a substantial amount of free DNA. If the DNA dose is limiting or if the calcium phosphate concentration is excessive, the antibody response to the DNA vaccine formulation may be inhibited.

TABLE 10

Adjuvant Effect of $CaPO_4$ vs $AlPO_4$ for HBs DNA or protein vaccines

Adjuvant	Dose (mg/mL)	Vaccine	Anti-HBs mIU/mL (Vaccine Dose) (1 µg)	(10 µg)	(100 µg)	% unbound DNA (@ 100 µg dose)
none	0	HBs DNA	1.9	111.0	313.0	n.a.
AlPO4	0.45		2.6	4,303.0	16,375.0	~100
CaPO4	10		4.0	4.5	556.0	7
	3.3		1.0	1.2	4,370.0	64
	1		1.0	7.2	1,782.0	92
	0.3		1.4	363.0	2,091.0	~100
	3.3	HBs protein		n.d.	n.d.	n.a.
			87,636.0			
Al(OH)PO4	0.45			n.d.	n.d.	n.a.
			105,240.0			

V1R.S Plasmid DNA

n = 10 BALB/c mice per group

Injection route: 0.05 mL in 2 intramuscular sites

Injection schedule: d. 0, 42

Assay time: d. 84

EXAMPLE 11
HBV DNA VACCINE / ALUMINUM PHOSPHATE FORMULATION AS A PRIMING ANTIGEN

A study comparing anti-HBs antibody production in mice primed with two doses of a hepatitis B DNA vaccine (V1Jns.S2.S at 100 μg per dose), (1) with aluminum phosphate, (2) without aluminum phosphate, and (3) Recombivax HB®. These priming immunogens were followed by either a boosting with either a V1Jns.S2.S or Recombivax HB®. Table 11 shows pre-boost and post-boost HBs antibody titers. DNA/AlPO4 priming followed by boosting with Recombivax HB® results in approximately a 300-fold increase in HBs antibody when compared to DNA priming (without aluminum phosphate) prior to boosting with Recombivax HB®.

TABLE 11

Effective priming with HBV DNA + $AlPO_4$ for a booster response to a conventional protein vaccine

			Anti-HBs GMT (mIU/mL)	
Priming Immunogen (2 doses)	Adjuvant	Booster	pre-boost	post-boost
DNA	none	DNA	14	39
		Protein		112
DNA	$AlPO_4$	DNA	279	307
		Protein		39,750
Protein	$Al(OH)PO_4$	Protein	11,100	106,606

DNA vaccine 100 μg per dose): V1Jns.S2.S

Protein vaccine (1 μg): Recombivax HB®

EXAMPLE 12

EFFECT OF ALUMINUM PHOSPHATE ON POTENCY OF A HERPES SIMPLEX DNA VACCINE IN GUINEA PIGS

Plasmids V1Jns:gD and V1Jns:ΔgB encoding HSV-2 glycoprotein D (gD) and the amino-terminal 707 amino acids of glucoprotein B (gB), respectively have been described in McClements et al. (1996, *Proc Natl Acad Sci U S A* 93: 11414-11420) . The vaccines were prepared by diluting V1Jns:gD DNA and V1Jns:ΔgB DNA into either sterile PBS, or sterile PBS containing AdjuPhos® at a final aluminum concentration of 450 μg/mL. Vaccines were thoroughly mixed by gentle vortexing then stored at 4°C for 24 hours. Immediately prior to injection, the vaccine formulations were subjected to gentle vortexing. Female Duncan Hartley guinea pigs (Harlan Sprague Dawley; Indianapolis, IN) weighing between 450-550 grams at the time of the first immunization were injected with a total of 200 μL (100 μL per quadriceps muscle) containing 6 μg V1Jns:gD + 20 μg V1Jns:ΔgB, with or without 90 μg aluminum. Animals were boosted at five weeks. Sera obtained at weeks 4 and 8 were assayed at ten-fold dilutions, ranging from 1:30 to 1:30,000, using gD- and gB-specific ELISAs (McClements et al, 1996, *Proc Natl Acad Sci U S A* 93: 11414-11420). Endpoint titers were determined as described previously except that serum dilutions were considered positive if the OD_{450} signal was > 0.05 above that of the preimmune sera at the same dilution (McClements et al, 1996, *Proc Natl Acad Sci U S A* 93: 11414-11420). These results are presented in Table 12.

TABLE 12

ELISA GMT; linear values (range) and $\log_{10}$ values ± SEM; N=4

group	4 wk		8 wk	
	anti gD	anti gB	anti gD	anti gB
DNA in PBS	17 (9-33)	53 (10-190)	53 (10-284)	5335 (2744-10370)
	1.23 ± .29	1.73 ± .55	1.73 ± .73	3.73 ± .29
DNA + $AlPO_4$	30 (10-89)	949 (251-3585)	169 (17-1634)	30000 (30K-30K)*
	1.48 ± .49	2.98 ± .58	2.23 ± .99	4.48 ± 0

* all strongly positive at highest dilution tested

EXAMPLE 13
EFFECT OF ALUMINUM PHOSPHATE ON POTENCY OF AN INFLUENZA DNA VACCINE IN PRIMATES

Rhesus monkeys - Groups of 5 young adult Rhesus of either sex were injected intramuscularly in both triceps muscles with 0.5 mL of a solution containing 500 mcg/mL of V1Jns-HA/Georgia plasmid encoding the HA from influenza A/Georgia/03/93 (H3N2), dissolved in phosphate-buffered saline or in phosphate-buffered saline with 500 mcg/mL or 1000 mcg/mL of aluminum phosphate adjuvant. A separate control group received HA DNA and aluminum in contralateral arms. Immunizations were given at 0 time and again at 8 weeks. Animals were bled at two week intervals and sera were tested for antibodies against A/Georgia/03/93 by hemagglutination inhibition (Figure 12A) and by ELISA (Figure 12B). Use of aluminum phosphate adjuvant in combination with the DNA increased antibody titers compared to animals that received DNA alone or DNA and adjuvant in contralateral arms. Repeated measures analysis of variance indicated that the pooled antibody titers of groups that received DNA mixed with aluminum were significantly higher than the pooled antibody titers of groups that received no aluminum or aluminum and DNA in contralateral arms ($P<0.05$).

Chimpanzee - Four adult chimpanzees of either sex were injected intramuscularly in one triceps muscle with a volume of 1.0 mL containing 500 mcg of V1Jns-HA/Georgia plasmid encoding the HA from influenza A/Georgia/03/93 (H3N2), dissolved in phosphate-buffered saline or in phosphate-buffered saline with 500 mcg of aluminum phosphate adjuvant. Immunizations were given at time 0 and at 6 and 12 weeks. Sera were collected at two week intervals and were assayed for antibodies by HI, virus neutralization, and ELISA. Table 13 shows that greater antibody responses were seen in the two animals given HA DNA with aluminum adjuvant, with 1/2 in the alum group having at least fourfold rises in HI antibody and 2/2 having fourfold rises in virus neutralization, while 0/2 animals given HA DNA alone exhibited these responses.

Table 13

Antibody Responses of Chimpanzees to HA DNA Vaccine ± Alum adjuvant

Treatment	Animal	Week	Hi Titer vs. A/Georgia	Hi Titer vs. A/Guang-dong	A/Georgia ELISA	A/Georgia Virus Neutralization
HA DNA	X019	0	5	5	25	20
		4	5	5	50	20
		6	5	5	25	10
		8	5	5	25	10
		10	5	5	200	20
		12	5	5	200	20
		14	10	5	200	40
HA DNA	X131	0	5	5	400	20
		4	5	5	200	20
		6	10	5	200	10
		8	5	5	200	20
		10	10	5	200	20
		12	5	5	200	20
		14	10	5	200	40
HA DNA + AlPO4	X133	0	5	5	50	10
		4	5	5	50	20
		6	10	5	100	20
		8	20	40	1600	160
		10	10	20	800	80
		12	10	20	400	80
		14	20	40	800	320
HA DNA + AlPO4	X140	0	5	5	100	10
		4	5	5	100	5
		6	5	5	200	ND
		8	10	5	200	20
		10	10	5	200	20
		12	5	5	100	10
		14	10	5	400	80

EXAMPLE 14

EFFECT OF ALUMINUM PHOSPHATE ON POTENCY OF AN INFLUENZA DNA VACCINE IN HUMANS

The V1Jns-HA/Georgia plasmid (IDV) encoding the HA from influenza A/Georgia/03/93 (H3N2) or placebo was administered with and without aluminum phosphate ($AlPO_4$) at varying dosages to investigate whether $AlPO_4$ would enhance immunogenicity. Seventy eight healthy subjects aged 18-45 were enrolled at a single site (Johns Hopkins University). Subjects with a hemagglutination inhibition (HI) titer >1/32 were excluded. Subjects received vaccine at day 0 and at 2 months and 6 months. This DNA vaccine, admininstered alone or in combination with $AlPO_4$ was generally well tolerated in healthy adults. The V1Jns-HA/Georgia plasmid dose ranged from 0 μg to 500 μg while the aluminum phosphate dose ranged from 0 μg to 700 μg in this particular study. As noted in this specification, it will be within the purview of the skilled artisan to utilize these alternative combinations of DNA and adjuvant when practicing the invention. Such alternative combinations should only be limited to physical parameters such as solubility, as well as the therapeutic and prophylactic affect to the patient. Table 14 shows the proportion of subjects which exhibited at least a four fold rise in antibody production 3 weeks after immunization. It is evident that the addition of 700 μg of $AlPO_4$ with the DNA vaccine enhanced the ability of the DNA vaccine to elicit HI and neutralizing antibody responses. This point is shown further in Table 15, which shows a comparison of geometric mean titers of HI and neutralizing antibody prior to a first dose (day 0) and 3 weeks after the second dose with V1Jns-HA/Georgia plasmid, with our without various $AlPO_4$ concentrations. Therefore, DNA vaccine formulations comprising nucleic acid molecules (such as a flu DNA vaccine) used in conjunction with an adjuvant which does not substantially bind the nucleic acid molecules (such as aluminum phosphate) results in a marked increase in an immune response of the host.

Table 14

Proportion of Subjects with ≥ 4 Fold rises in Antibody Responses
3 weeks after Immunization with V1Jns-HA/Georgia (IVD) with or without $A1PO_4$

Vaccine Dose µg		HI Antibody Rises				Neutralizing Antibody	
		After 1st Dose		After 2nd Dose*		After 2nd Dose*	
IDV	$A1PO_4$	GA	Guangdong	GA	Guangdong	GA	Guangdong
500	700	0/15	3/15	2/13	5/13	5/13	8/13
300	700	0/10	4/10	1/8	7/8	3/8	6/8
100	700	2/10	4/10	2/8	5/8	2/8	5/8
300	450	0/10	1/10	1/10	6/10	1/10	5/10
300	225	0/10	1/10	1/9	2/9	0/9	2/9
300	0	0/10	0/10	0/10	1/10	0/10	0/10
0	700	0/13	0/13	0/11	0/11	0/11	0/11

* Data from subjects with possible Flu were excluded.

Table 15

Geometric Mean Titers of HI and Neutralizing Antibody
Before and After 2 Doses of V1Jns-HA/Georgia (IVD) with or without $AlPO_4$

Vaccine			HI Antibody				Neutralizing Antibody			
Dose µg			GA		Guangdong		GA		Guangdong	
IDV	$AlPO_4$	N	Day 0	3 wks. post dose 2*	Day 0	3 wks. post dose 2*	Day 0	3 wks. post dose 2*	Day 0	3 wks. post dose 2*
500	700	15	23	38[b]	6	12[b]	42[a]	84[b]	23[a]	68[b]
300	700	10	32	76[c]	6	45[c]	54[d]	160[c]	34[d]	226[c]
100	700	10	30	54[c]	8	16[c]	47[d]	73[c]	22[d]	67[c]
300	450	10	45	79	11	28	65	98	40	86
300	225	10	26	44[d]	6	10[d]	37	43[d]	23	40[d]
300	0	10	69	74	20	24	80	70	75	70
0	700	13	42	44[c]	12	12[c]	104	75[c]	68	45[c]

*Data from subjects with possible Flu were excluded.
Subjects evaluable at timepoint of interest. N= [a]14, [b]13, [c]11, [d]9, [e]8

EXAMPLE 15

ENHANCED CYTOKINE-SECRETING T CELL RESPONSES TO HBs DNA VACCINE FORMULATED WITH ALUMINUM PHOSPHATE

For this Example, mice were generally 6 - 8 weeks of age at the start of experiments. Adjuvant concentration was calculated on the basis of calcium or aluminum content. Vaccine formulations were prepared by mixing plasmid DNA (in saline) with various concentrations of calcium or aluminum phosphate in 0.85% NaCl within 1 hr of injection. The HBs DNA constructs utilized in this Example are described in Example 7. The hepatitis B surface antigen (HBsAg) used herein is derived from *Saccharomyces cerevisiae* containing the gene for the *adw* subtype of HbsAg. In certain experiments the HBsAg was formulated with aluminum hydroxide adjuvant at 10 - 20 μg HBsAg per 450 μg aluminum per mL.

ELISPOT assay for cytokine production. Spleen cells from immunized mice were assayed for the ability to secrete IFN-γ or IL-2 during in vitro restimulation with antigenic peptides by a modification of previous methods [25-27] . Briefly, 96-well polyvinylidine difluoride (PVDF)-backed plates (MAIP NOB 10; Millipore, Bedford, MA) were coated with antibody to (IFN-γ) (clone R4-6A2 Pharmingen #18181D) or IL-2 (clone JES6-1A12 Pharmingen #18161D), washed three times with PBS, and then blocked with RPMI-1640 medium containing 10% heat-inactivated FBS. Cells were cultured at 5 x 10^5 per well in 0.1 mL of medium for restimulation with defined peptides corresponding to a known CTL epitope (IPQSLDSWWTSL) or with a newly identified helper epitope (NCTCIPIPSSWAFGK). After 18-24 hr incubation at 37° C, the plates were washed 6 times with PBS containing 0.005% Tween 20. Developing antibodies were biotinylated anti-mouse (IFN-γ), clone XMG1.2 (Pharmingen #18112D) or biotin-labled anti-mouse IL-2 clone JES6-5H4 (Pharmingen #18172D). The plates were washed 6 more times before the addition of Streptavidin-HRP conjugate (Southern Biotech Assoc., Birmingham, AL; #7100-05). After 3 washes with PBS-Tween and 3 washes with PBS, spots were developed with 3-amino-9-ethylcarbazole (Sigma #A6926). Spots were counted using a stereomicroscope.

The ELISPOT assay was used to determine the number of T cells secreting interferon-γ (IFN-γ) or IL-2 upon in vitro restimulation with antigenic peptides. BALB/c mice were immunized with 10 μg of V1R.S DNA ± aluminum phosphate on day 0 and 21. Eight days later, spleen cells were harvested for the ELISPOT assay. The IFN-γ response was elicited by overnight culture with HBs

peptide [28-39] which, in addition to induction of CTLs, stimulates CD8 T cells to produce IFN-γ. As shown in Figure 13A, mice immunized with HBV DNA + aluminum phosphate generated ~ 5-fold more IFN-γ ELISPOTs than did mice immunized with the DNA vaccine alone. Furthermore, the affinity of T cells appeared to be increased since detectable responses could be elicited with 5-10 fold lower concentrations of peptide in mice immunized with HBV DNA + aluminum phosphate compared to mice immunized with naked DNA. Similarly, immunization with HBV + aluminum phosphate elicited a stronger IL-2 ELISPOT response than did injection of HBV DNA alone (Figure 13B). The IL-2 response was elicited with a 15mer HBs peptide [146-160] that had been identified by screening a set of 15mer peptides (offset by 1 amino acid) comprising the entire HBs protein. The response to this peptide is mediated by CD4 T cells (data not shown). As seen with the IFN-γ ELISPOT response, immunization with HBV DNA + aluminum phosphate appears to elicit T cells with a higher affinity for the 15mer peptide than did immunization with HBV DNA alone. Interestingly, the adjuvant also improved the quality of the ELISPOTs: both the size and intensity of spots was increased for IFN-γ and IL-2 T cell responses to the HBV DNA vaccine.

By using the more sensitive ELISPOT assay to measure T cell responses to DNA ± aluminum phosphate, this data exemplifies an enhanced cytokine response to HBV peptide antigens. In order to assess TH1 cytokine responses to HBsAg, it was necessary to first identify a CD4 T cell epitope since none had been reported previously. This was accomplished by screening an HBs pepset consisting of overlapping 15mers encompassing the entire HBs protein sequence resulting in the identification of HBs [146-160] as a TH epitope capable of eliciting an IL-2 response in HBV DNA vaccine-immune mice. Immunization of BALB/c mice with HBV DNA formulated with aluminum phosphate resulted in an increase in both the number and apparent affinity of T cells producing IL-2 upon restimulation with this peptide in the ELISPOT assay. Similarly, HBV DNA + aluminum phosphate enhanced the number and affinity of the IFN-γ T cell response to the known CTL epitope, HBs [28-39]. TH2 cytokine responses, including IL-4 and IL-5, were not detected in mice immunized with HBV DNA ± aluminum phosphate. Consistent with this finding, the isotype profile of the antibody response is also characteristic of a TH1 response, and boosting with the adjuvanted DNA vaccine preferentially augments this response. Thus, this example shows that "alum" adjuvants can enhance the potency of DNA

vaccines without affecting their unique ability to induce cell mediated immunity characteristic of highly efficacious live-attenuated virus vaccines.

5

WHAT IS CLAIMED:

1. A pharmaceutical formulation, comprising:

(a) a mineral-based, negatively charged adjuvant; and,

(b) a polynucleotide vaccine encoding at least one antigen, such that introduction of said formulation into a vertebrate host results in expression of a biologically effective amount of said antigen or antigens so as to induce a prophylactic or therapeutic immune response.

2. A pharmaceutical formulation of claim 1 wherein said mineral adjuvant is an aluminum phosphate-based adjuvant.

3. A pharmaceutical formulation of claim 2 wherein the molar PO_4/Al ratio of said aluminum phosphate-based adjuvant does not substantially bind to nucleic acid molecules.

4. A pharmaceutical formulation of claim 3 wherein said molar PO_4/Al ratio is about 0.9.

5. A pharmaceutical formulation of claim 3 wherein said aluminum-phosphate based adjuvant is Adju-Phos®.

6. A pharmaceutical formulation of claim 4 wherein said aluminum-phosphate based adjuvant is Adju-Phos®.

7. A pharmaceutical formulation of claim 5 wherein said polynucleotide vaccine expresses said antigen or antigens so as to induce a prophylactic or therapeutic immune response against a disease or disorder selected from the group consisting of human immunodeficiency virus, herpes simplex virus, human influenza, hepatitis A, hepatitis B, hepatitis C, human papilloma virus, tuberculosis, tumor growth, autoimmune disorders and allergies.

8. A pharmaceutical formulation of claim 6 wherein said polynucleotide vaccine expresses said antigen or antigens so as to induce a prophylactic or therapeutic immune response against a disease or disorder selected from the group consisting of human immunodeficiency virus, herpes simplex virus, human influenza, hepatitis A, hepatitis B, hepatitis C, human papilloma virus, tuberculosis, tumor growth, autoimmune disorders and allergies.

9. A pharmaceutical formulation of claim 5 wherein said polynucleotide vaccine expresses said antigen or antigens so as to induce a prophylactic or therapeutic immune response against a veterinary disease or disorder selected from the group consisting of rabies, distemper, foot and mouth disease, anthrax, bovine herpes simplex and bovine tuberculosis.

10. A pharmaceutical formulation of claim 6 wherein said polynucleotide vaccine expresses said antigen or antigens so as to induce a prophylactic or therapeutic immune response against a veterinary disease or disorder selected from the group consisting of rabies, distemper, foot and mouth disease, anthrax, bovine herpes simplex and bovine tuberculosis.

11. A pharmaceutical formulation of claim 7 wherein said polynucleotide vaccine is a DNA plasmid.

12. A pharmaceutical formulation of claim 8 wherein said polynucleotide vaccine is a DNA plasmid.

13. A pharmaceutical formulation of claim 9 wherein said polynucleotide vaccine is a DNA plasmid.

14. A pharmaceutical formulation of claim 10 wherein said polynucleotide vaccine is a DNA plasmid.

15. A method of inducing an immune response in an vertebrate host which comprises introducing the pharmaceutical formulation of claim 3 into said vertebrate host.

16. A method of inducing an immune response in an vertebrate host which comprises introducing the pharmaceutical formulation of claim 4 into said vertebrate host.

17. A method of inducing an immune response in an vertebrate host which comprises introducing the pharmaceutical formulation of claim 5 into said vertebrate host.

18. A method of inducing an immune response in an vertebrate host which comprises introducing the pharmaceutical formulation of claim 6 into said vertebrate host.

19. The method of claim 15 wherein introduction of said pharmaceutical formulation is introduced into said host as selected from the group consisting of parenteral, inhalation, and oral delivery.

20. The method of claim 16 wherein introduction of said pharmaceutical formulation is introduced into said host as selected from the group consisting of parenteral, inhalation, and oral delivery.

21. The method of claim 17 wherein introduction of said pharmaceutical formulation is introduced into said host as selected from the group consisting of parenteral, inhalation, and oral delivery.

22. The method of claim 18 wherein introduction of said pharmaceutical formulation is introduced into said host as selected from the group consisting of parenteral, inhalation, and oral delivery.

23. The method of claim 19 wherein said method of introduction is intramuscular.

24. The method of claim 20 wherein said method of introduction is intramuscular.

25. The method of claim 21 wherein said method of introduction is intramuscular.

26. The method of claim 22 wherein said method of introduction is intramuscular.

27. A pharmaceutical formulation of claim 1 wherein said mineral adjuvant is a calcium phosphate-based adjuvant.

28. A pharmaceutical formulation of claim 27 wherein said polynucleotide vaccine expresses said antigen or antigens so as to induce a prophylactic or therapeutic immune response against a disease or disorder selected from the group consisting of human immunodeficiency virus, herpes simplex virus, human influenza, hepatitis A, hepatitis B, hepatitis C, human papilloma virus, tuberculosis, tumor growth, autoimmune disorders and allergies.

29. A pharmaceutical formulation of claim 27 wherein said polynucleotide vaccine expresses said antigen or antigens so as to induce a prophylactic or therapeutic immune response against a veterinary disease or disorder selected from the group consisting of rabies, distemper, foot and mouth disease, anthrax, bovine herpes simplex and bovine tuberculosis.

30. A pharmaceutical formulation of claim 28 wherein said polynucleotide vaccine is a DNA plasmid.

31. A pharmaceutical formulation of claim 29 wherein said polynucleotide vaccine is a DNA plasmid.

32. A method of inducing an immune response in an vertebrate host which comprises introducing the pharmaceutical formulation of claim 27 into said vertebrate host.

33. A method of inducing an immune response in an vertebrate host which comprises introducing the pharmaceutical formulation of claim 28 into said vertebrate host.

34. A method of inducing an immune response in an vertebrate host which comprises introducing the pharmaceutical formulation of claim 29 into said vertebrate host.

35. The method of claim 32 wherein introduction of said pharmaceutical formulation is introduced into said host as selected from the group consisting of intramuscular, inhalation, and oral delivery.

36. The method of claim 33 wherein introduction of said pharmaceutical formulation is introduced into said host as selected from the group consisting of intramuscular, inhalation, and oral delivery.

37. The method of claim 34 wherein introduction of said pharmaceutical formulation is introduced into said host as selected from the group consisting of intramuscular, inhalation, and oral delivery.

38. The method of claim 35 wherein said method of introduction is intramuscular.

39. The method of claim 36 wherein said method of introduction is intramuscular.

40. The method of claim 37 wherein said method of introduction is intramuscular.

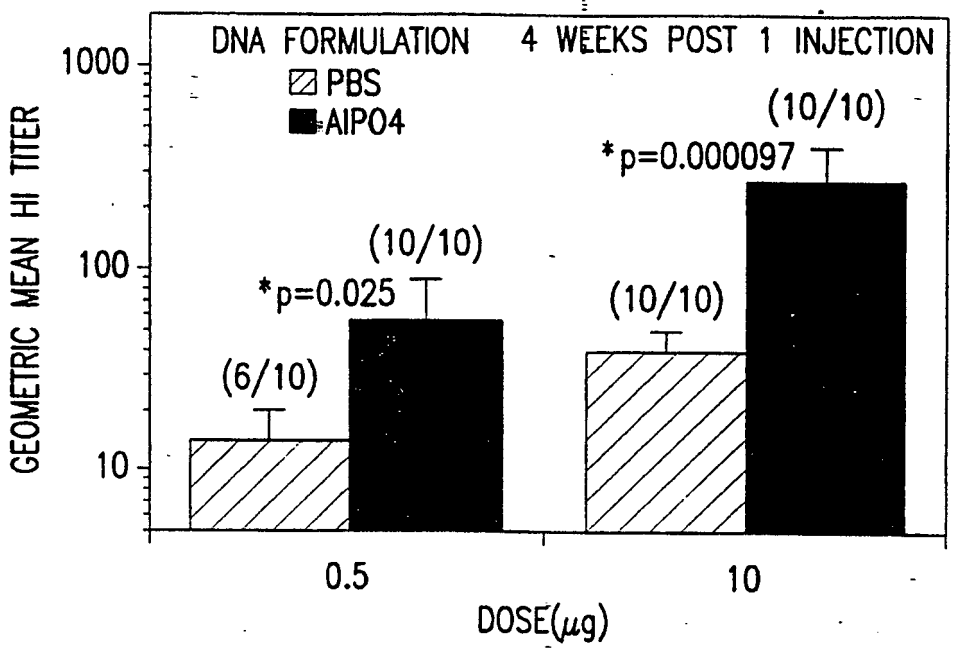
DNA FORMULATION 4 WEEKS POST 1 INJECTION
PBS
AlPO4
GEOMETRIC MEAN HI TITER
1000
100
10
(6/10)
(10/10)
*p=0.025
(10/10)
(10/10)
*p=0.000097
0.5
10
DOSE(μg)

FIG.1A

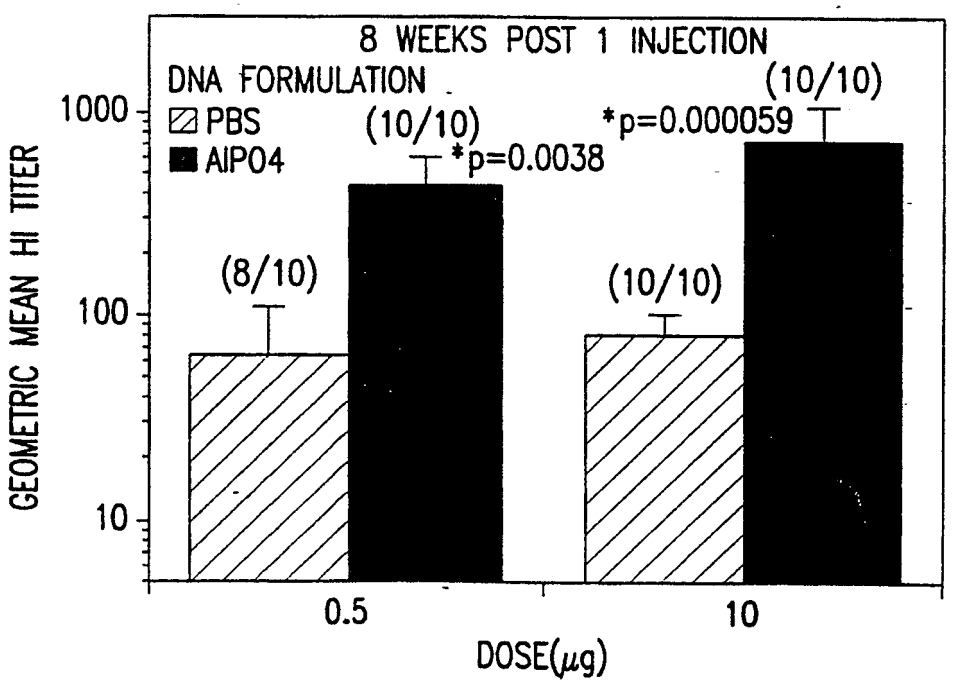
8 WEEKS POST 1 INJECTION
DNA FORMULATION
PBS
AlPO4
GEOMETRIC MEAN HI TITER
1000
100
10
(8/10)
(10/10)
*p=0.0038
(10/10)
(10/10)
*p=0.000059
0.5
10
DOSE(μg)

FIG.1B

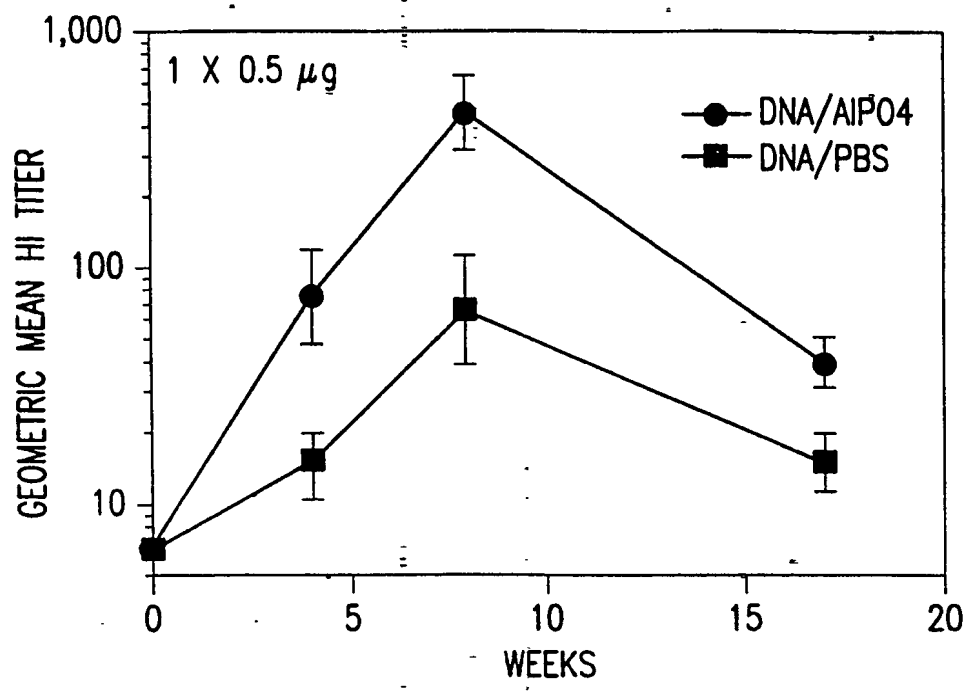
1,000
100
10
GEOMETRIC MEAN HI TITER
1 X 0.5 μg
DNA/AlPO4
DNA/PBS
0
5
10
15
20
WEEKS

FIG.2A

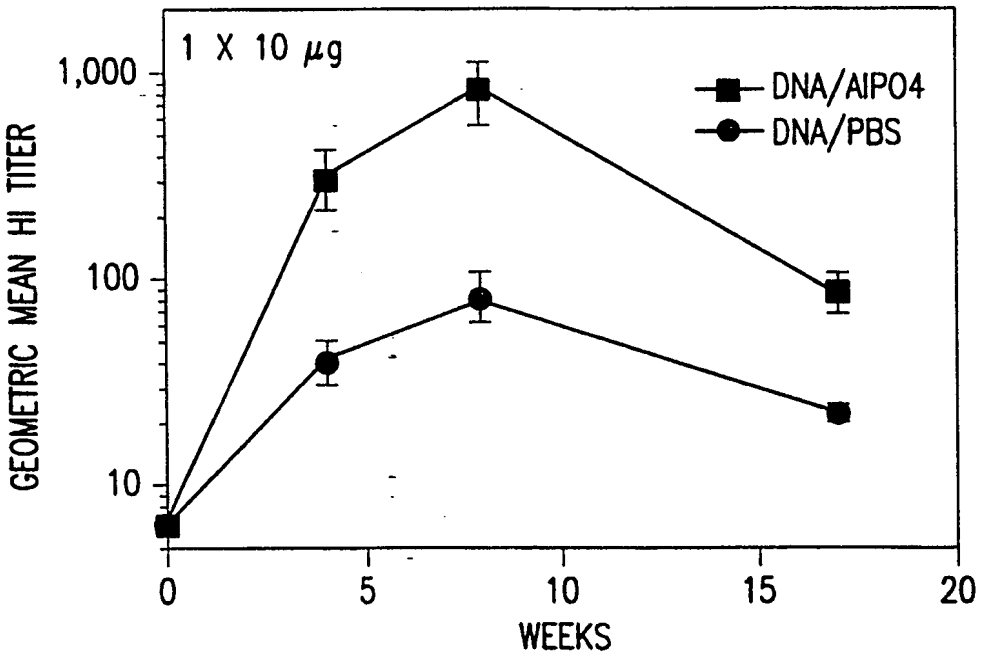
1,000
100
10
GEOMETRIC MEAN HI TITER
1 X 10 μg
DNA/AlPO4
DNA/PBS
0
5
10
15
20
WEEKS

FIG.2B

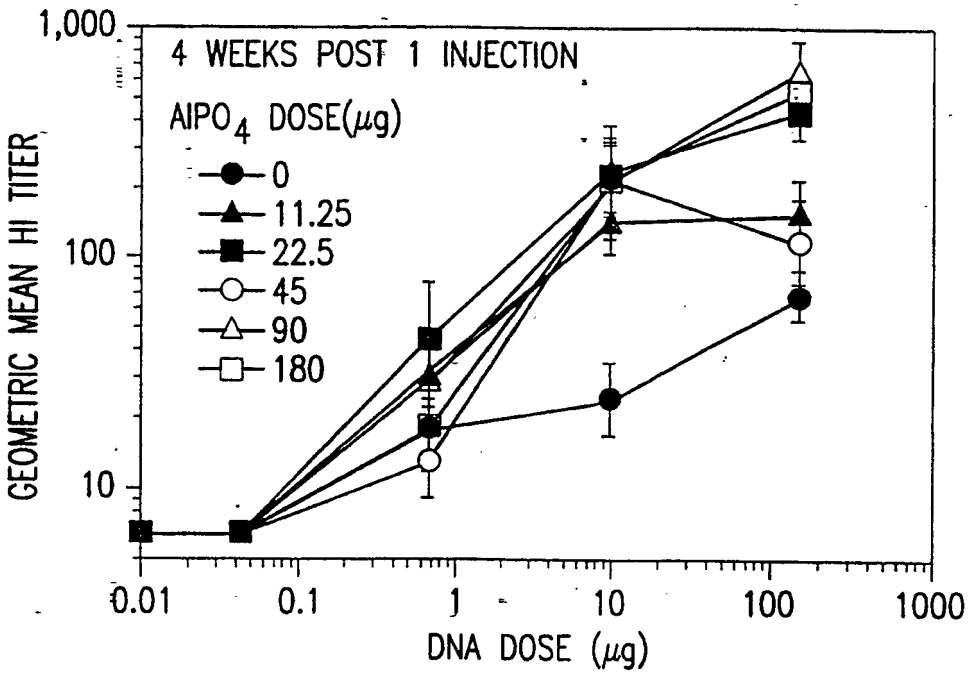
4 WEEKS POST 1 INJECTION
AlPO4 DOSE(μg)
0
11.25
22.5
45
90
180
GEOMETRIC MEAN HI TITER
1,000
100
10
0.01
0.1
1
10
100
1000
DNA DOSE (μg)

FIG.3A

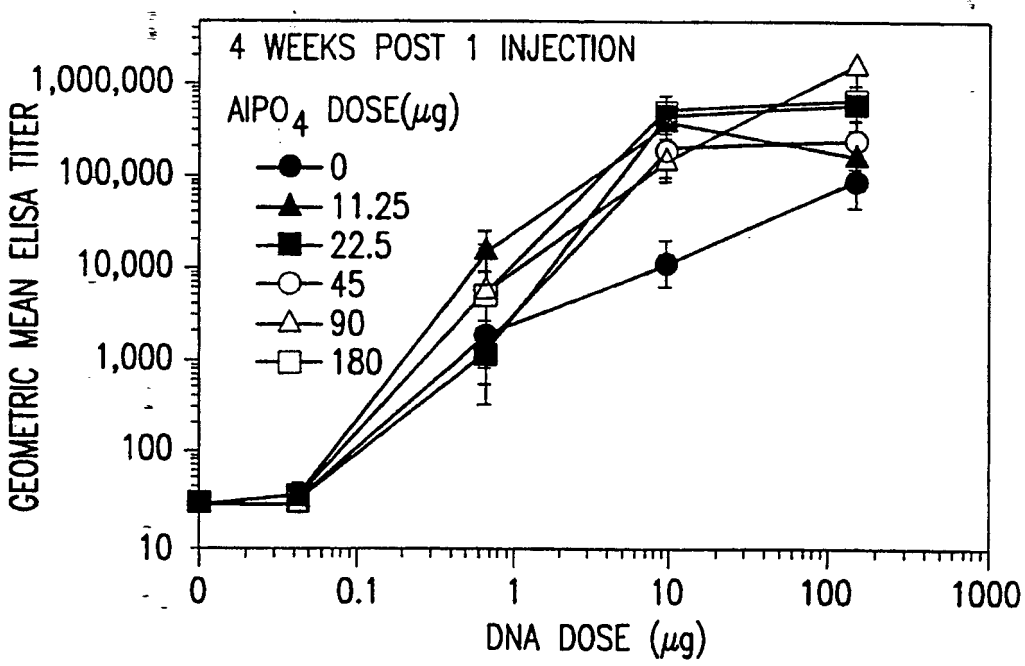
4 WEEKS POST 1 INJECTION
AlPO4 DOSE(μg)
0
11.25
22.5
45
90
180
GEOMETRIC MEAN ELISA TITER
1,000,000
100,000
10,000
1,000
100
10
0
0.1
1
10
100
1000
DNA DOSE (μg)

FIG.3B

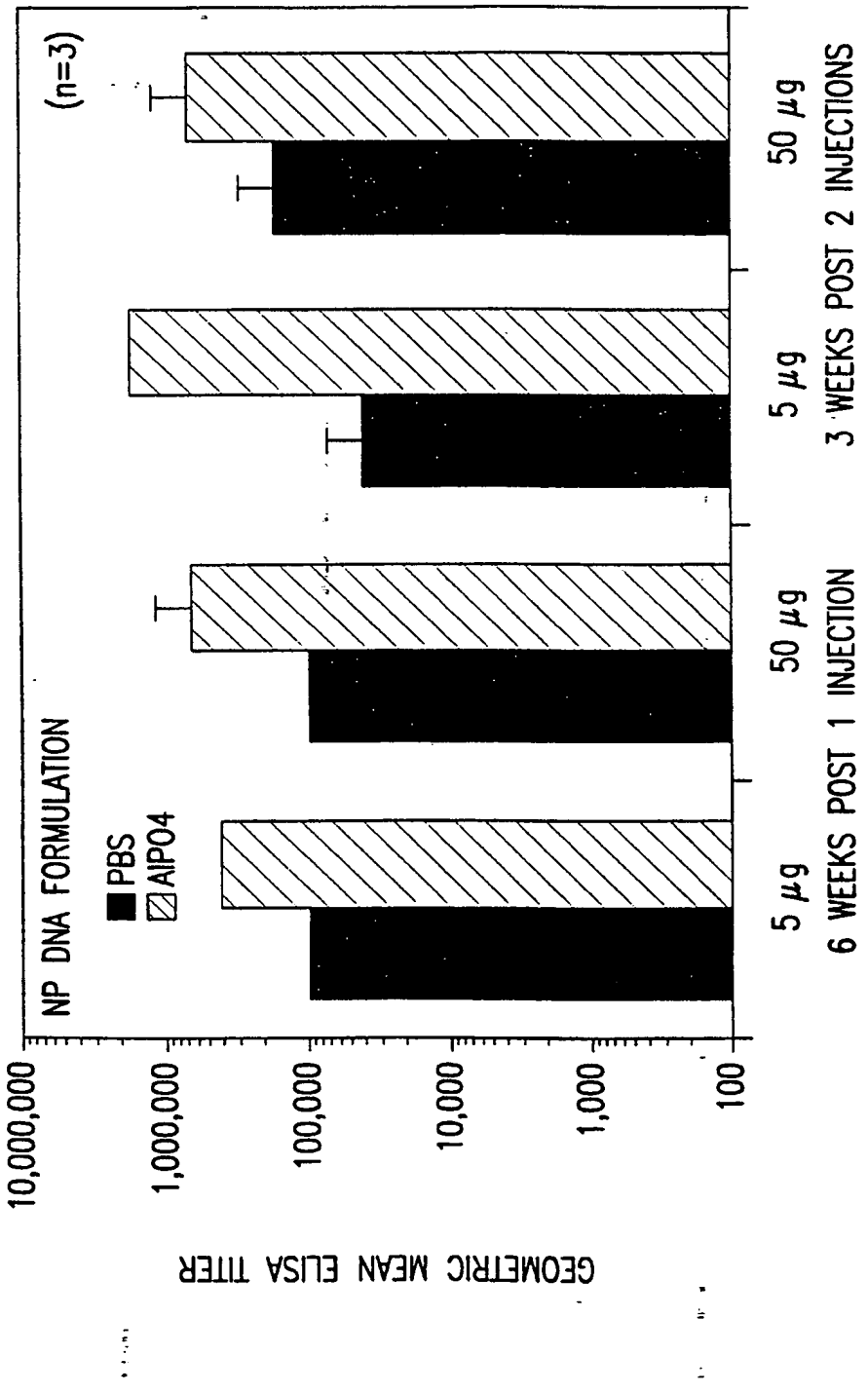
GEOMETRIC MEAN ELISA TITER
10,000,000
1,000,000
100,000
10,000
1,000
100
NP DNA FORMULATION
PBS
AIPO4
(n=3)
5 μg
50 μg
6 WEEKS POST 1 INJECTION
5 μg
50 μg
3 WEEKS POST 2 INJECTIONS

FIG.4

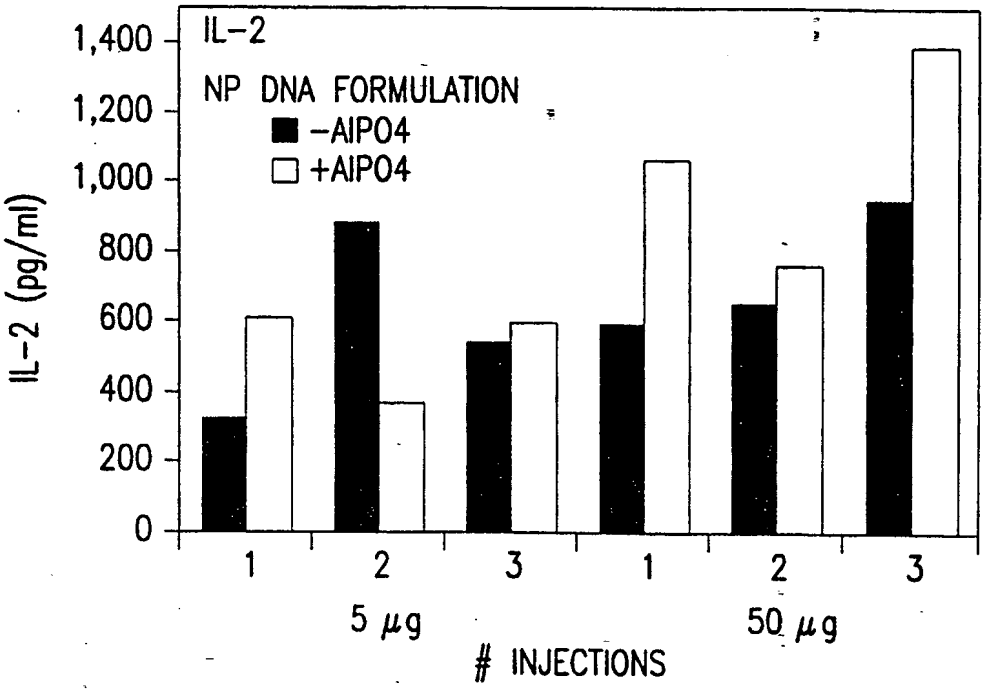
IL-2
NP DNA FORMULATION
-AlPO4
+AlPO4
IL-2 (pg/ml)
1,400
1,200
1,000
800
600
400
200
0
1
2
3
1
2
3
5 μg
50 μg
INJECTIONS

FIG.5A

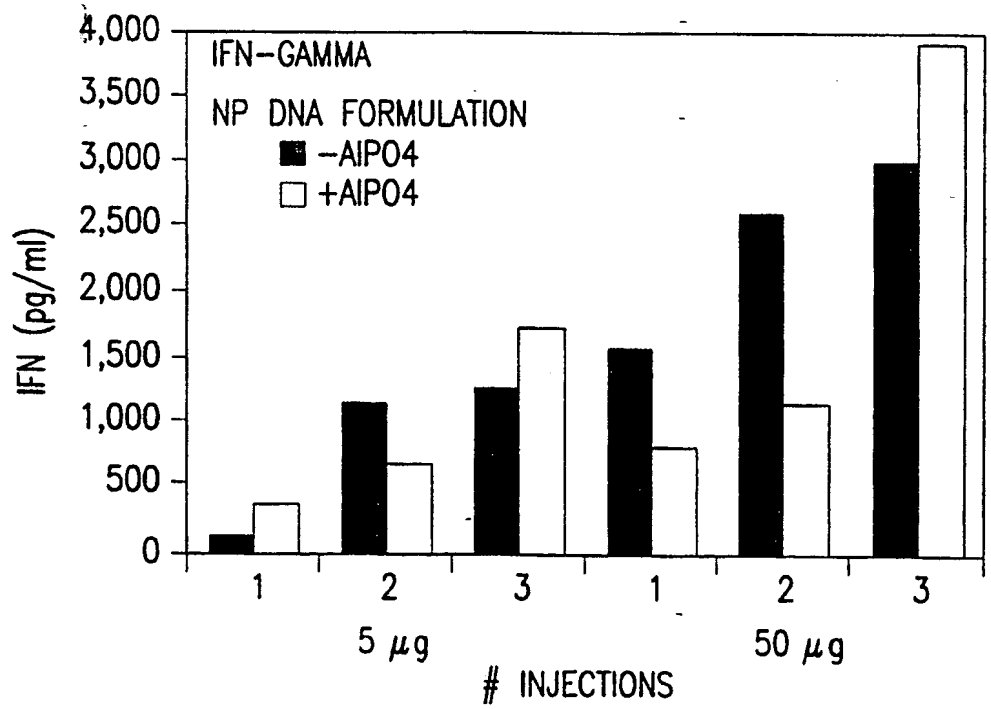
IFN-GAMMA
NP DNA FORMULATION
-AlPO4
+AlPO4
IFN (pg/ml)
4,000
3,500
3,000
2,500
2,000
1,500
1,000
500
0
1
2
3
1
2
3
5 μg
50 μg
INJECTIONS

FIG.5B

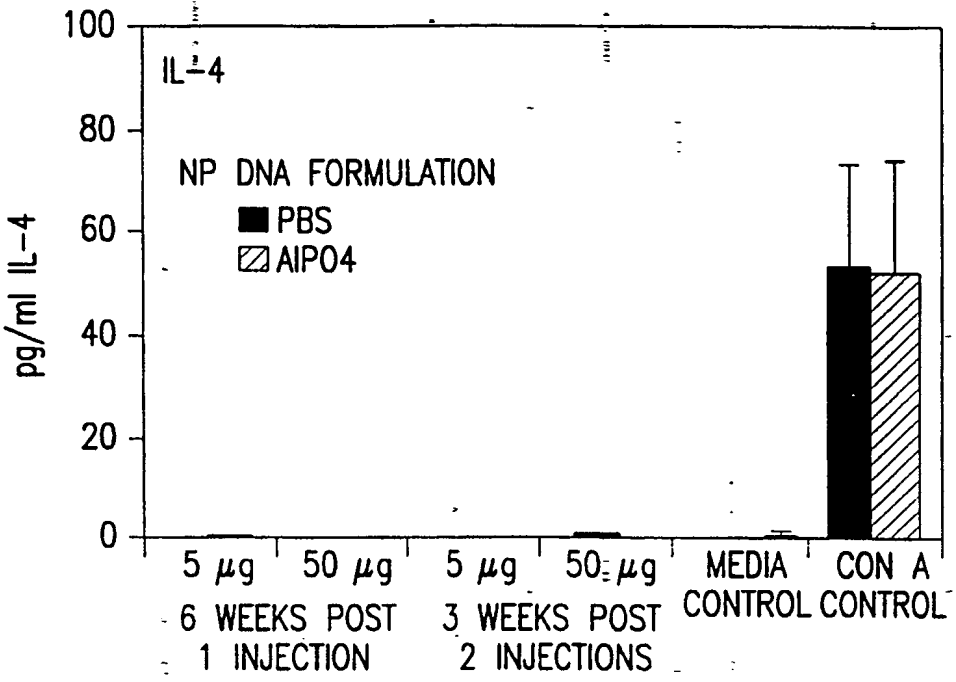
IL-4
NP DNA FORMULATION
PBS
AlPO4
pg/ml IL-4
100
80
60
40
20
0
5 μg
50 μg
5 μg
50 μg
MEDIA CONTROL
CON A CONTROL
6 WEEKS POST 1 INJECTION
3 WEEKS POST 2 INJECTIONS

FIG.5C

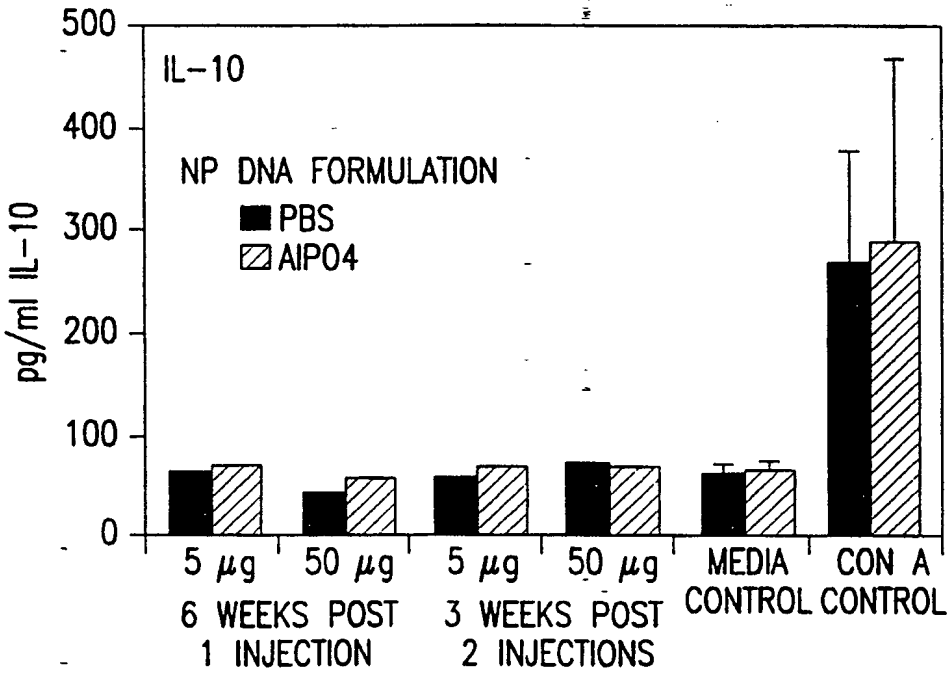
IL-10
NP DNA FORMULATION
PBS
AlPO4
pg/ml IL-10
500
400
300
200
100
0
5 μg
50 μg
5 μg
50 μg
MEDIA CONTROL
CON A CONTROL
6 WEEKS POST 1 INJECTION
3 WEEKS POST 2 INJECTIONS

FIG.5D

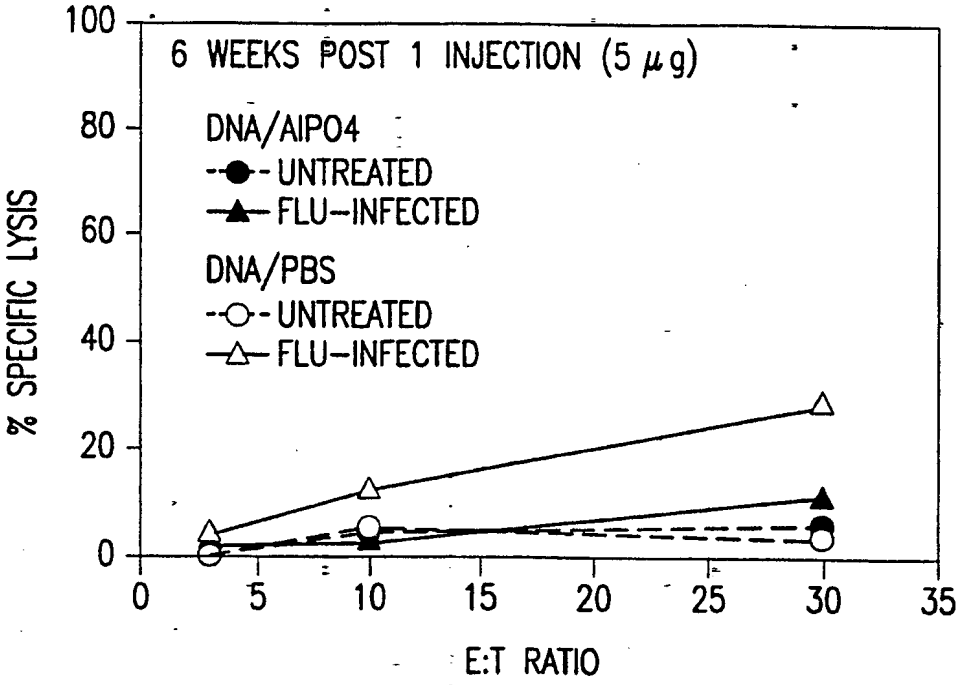
6 WEEKS POST 1 INJECTION (5 μg)
DNA/AIPO4
UNTREATED
FLU-INFECTED
DNA/PBS
UNTREATED
FLU-INFECTED
% SPECIFIC LYSIS
E:T RATIO
0
20
40
60
80
100
0
5
10
15
20
25
30
35

FIG.6A

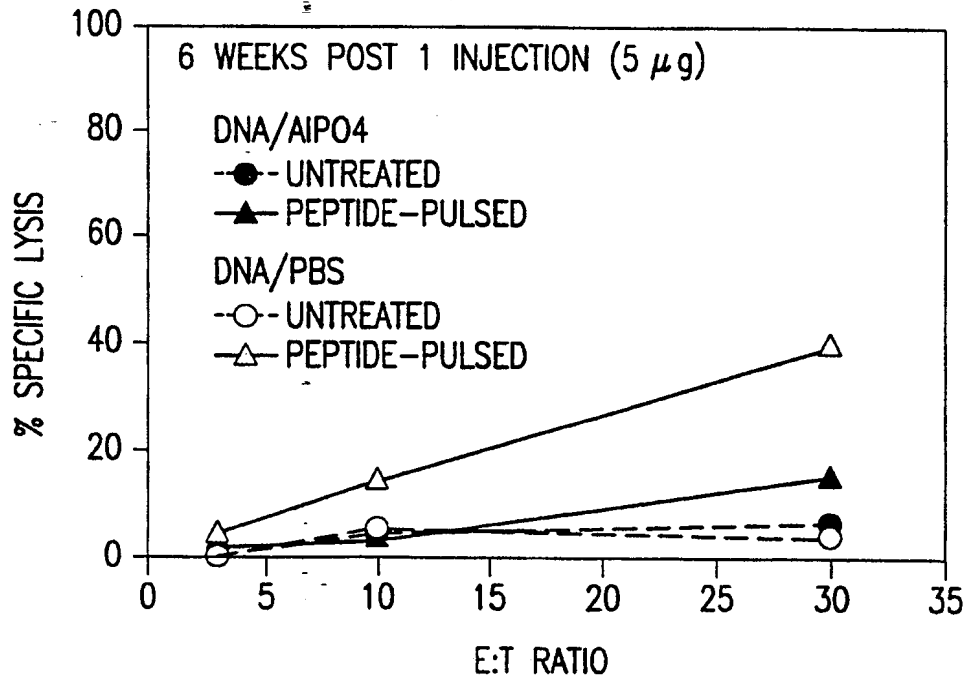
6 WEEKS POST 1 INJECTION (5 μg)
DNA/AIPO4
UNTREATED
PEPTIDE-PULSED
DNA/PBS
UNTREATED
PEPTIDE-PULSED
% SPECIFIC LYSIS
E:T RATIO
0
20
40
60
80
100
0
5
10
15
20
25
30
35

FIG.6B

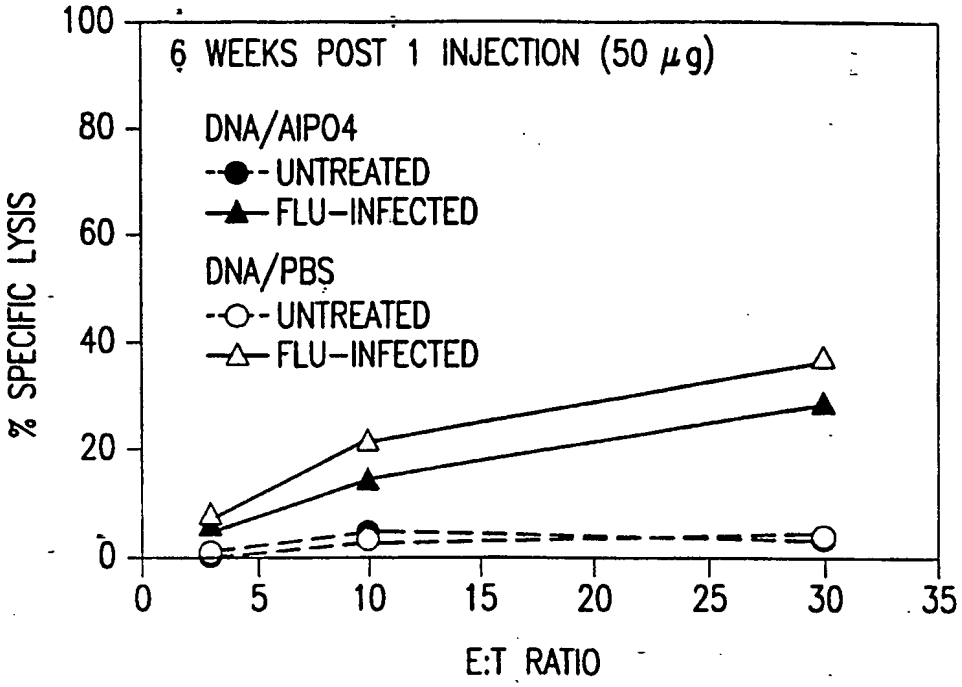
6 WEEKS POST 1 INJECTION (50 μg)
DNA/AIPO4
UNTREATED
FLU-INFECTED
DNA/PBS
UNTREATED
FLU-INFECTED
% SPECIFIC LYSIS
E:T RATIO
0
20
40
60
80
100
0
5
10
15
20
25
30
35

FIG.6C

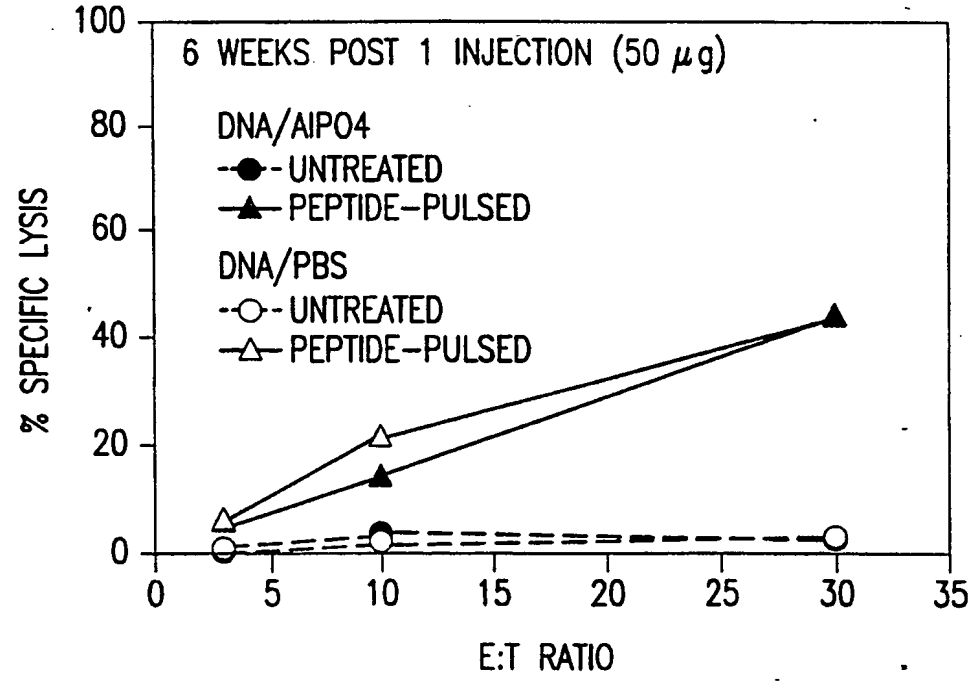
6 WEEKS POST 1 INJECTION (50 μg)
DNA/AIPO4
UNTREATED
PEPTIDE-PULSED
DNA/PBS
UNTREATED
PEPTIDE-PULSED
% SPECIFIC LYSIS
E:T RATIO
0
20
40
60
80
100
0
5
10
15
20
25
30
35

FIG.6D

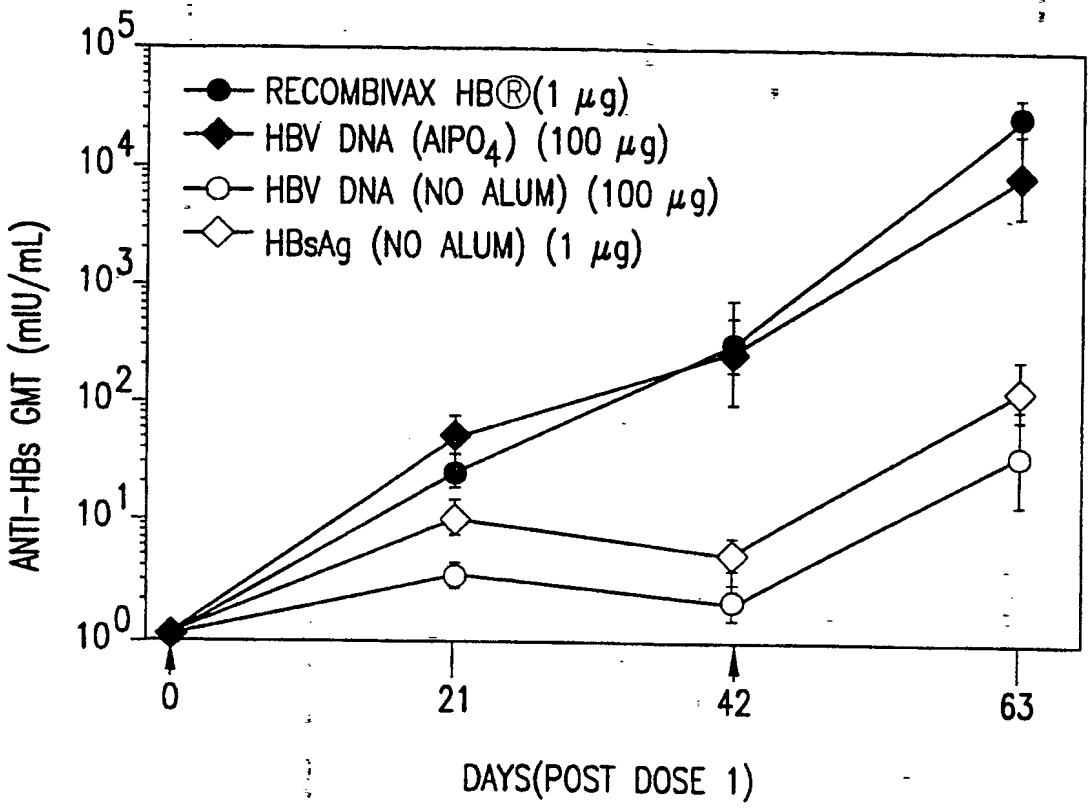
RECOMBIVAX HB®(1 μg)
HBV DNA ($AlPO_4$) (100 μg)
HBV DNA (NO ALUM) (100 μg)
HBsAg (NO ALUM) (1 μg)
ANTI-HBs GMT (mIU/mL)
10^5
10^4
10^3
10^2
10^1
10^0
0
21
42
63
DAYS(POST DOSE 1)

FIG.7

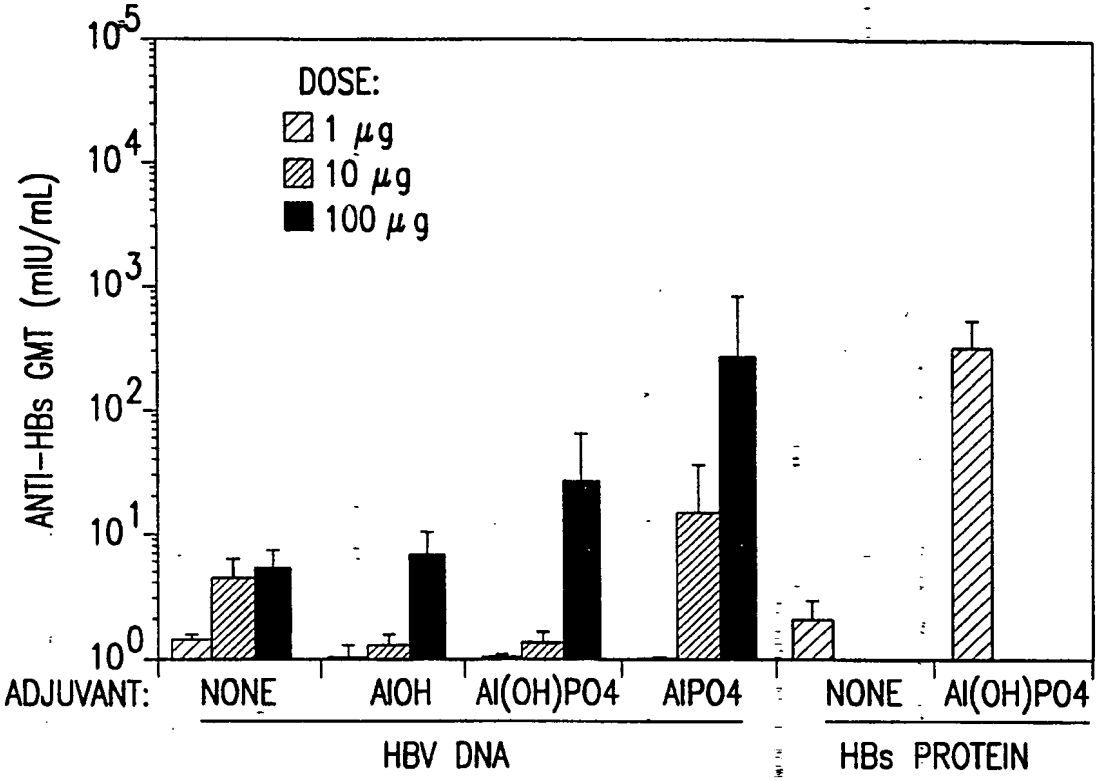
ANTI-HBs GMT (mIU/mL)
10^5
10^4
10^3
10^2
10^1
10^0
DOSE:
1 μg
10 μg
100 μg
ADJUVANT:
NONE
AlOH
Al(OH)PO4
AlPO4
NONE
Al(OH)PO4
HBV DNA
HBs PROTEIN

FIG.8

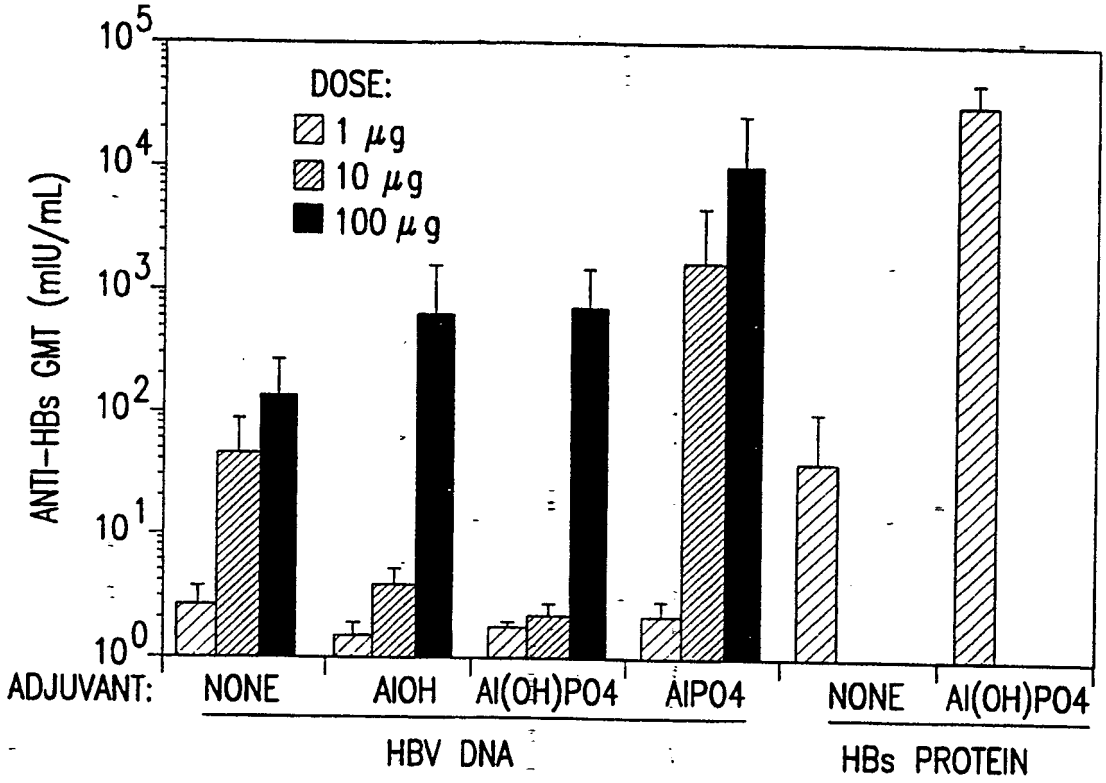
ANTI-HBs GMT (mIU/mL)
10^5
10^4
10^3
10^2
10^1
10^0
DOSE:
1 μg
10 μg
100 μg
ADJUVANT:
NONE
AlOH
Al(OH)PO4
AlPO4
NONE
Al(OH)PO4
HBV DNA
HBs PROTEIN

FIG.9

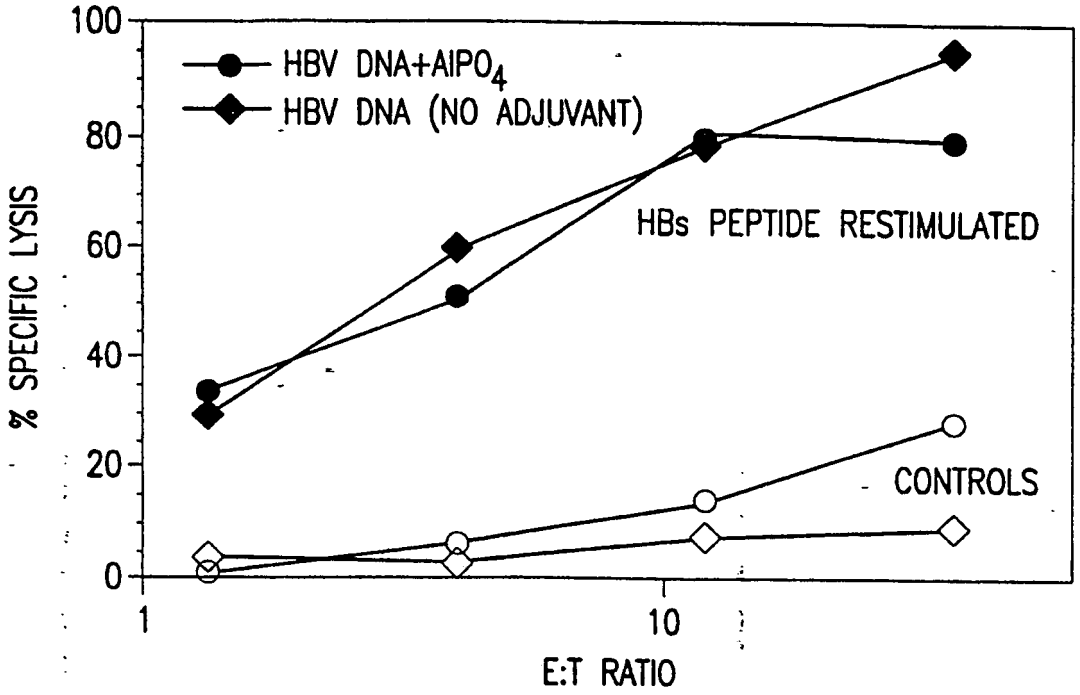
100
80
60
40
20
0
% SPECIFIC LYSIS
HBV DNA+AlPO4
HBV DNA (NO ADJUVANT)
HBs PEPTIDE RESTIMULATED
CONTROLS
1
10
E:T RATIO

FIG.10

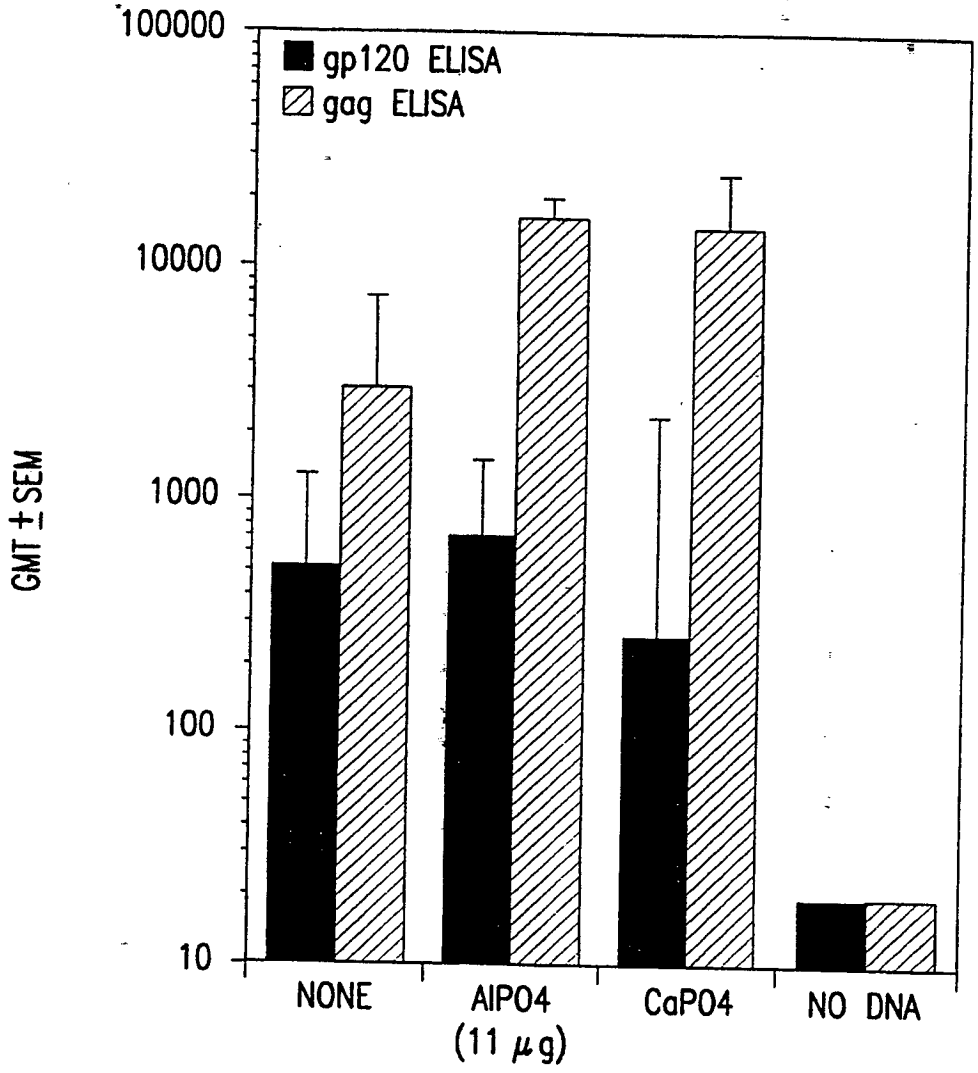
100000
10000
1000
100
10
GMT ± SEM
gp120 ELISA
gag ELISA
NONE
AlPO4
(11 μg)
CaPO4
NO DNA

FIG.11

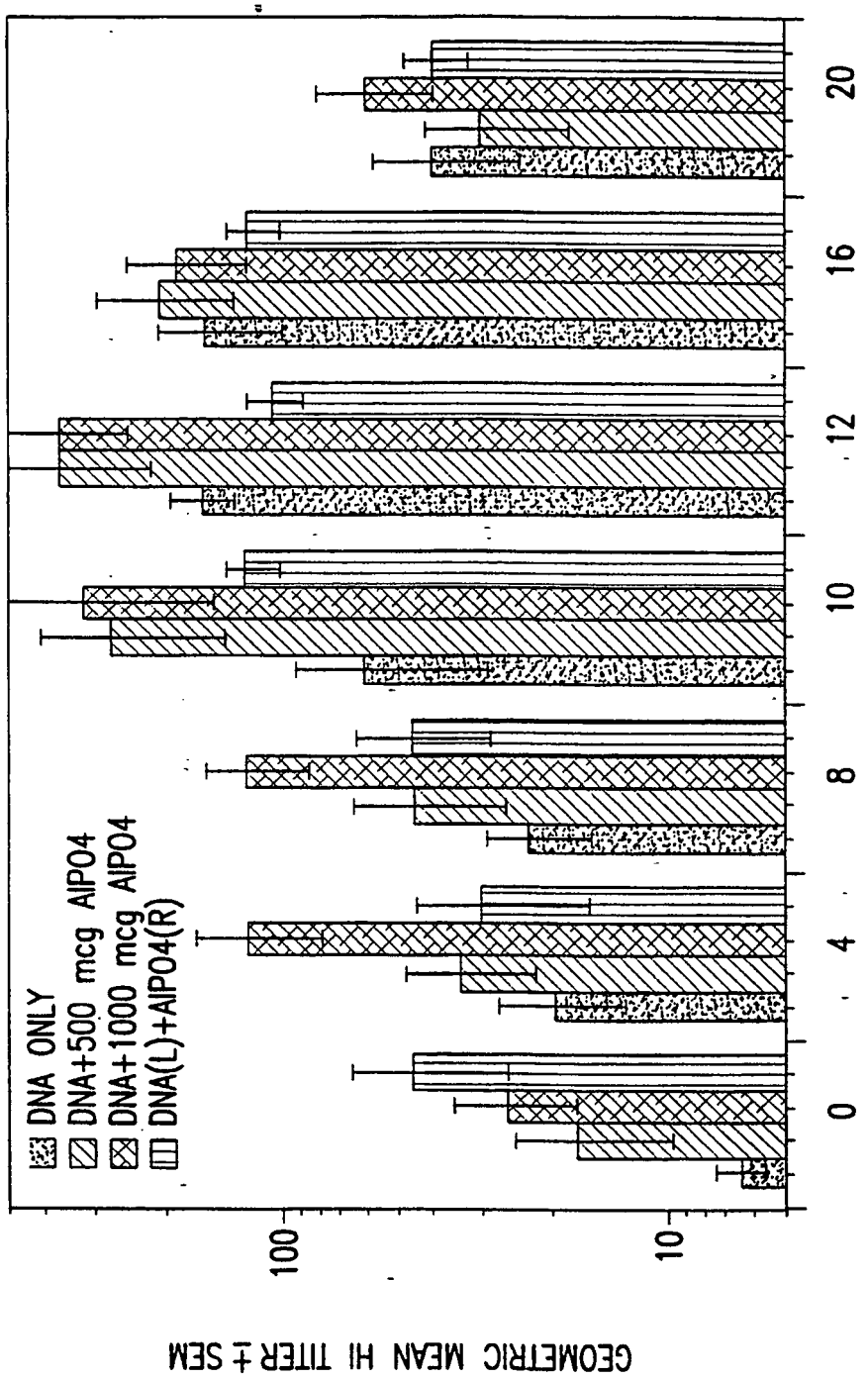
DNA ONLY
DNA+500 mcg AlPO4
DNA+1000 mcg AlPO4
DNA(L)+AlPO4(R)
100
10
0
4
8
10
12
16
20
WEEK
GEOMETRIC MEAN HI TITER ± SEM

FIG.12A

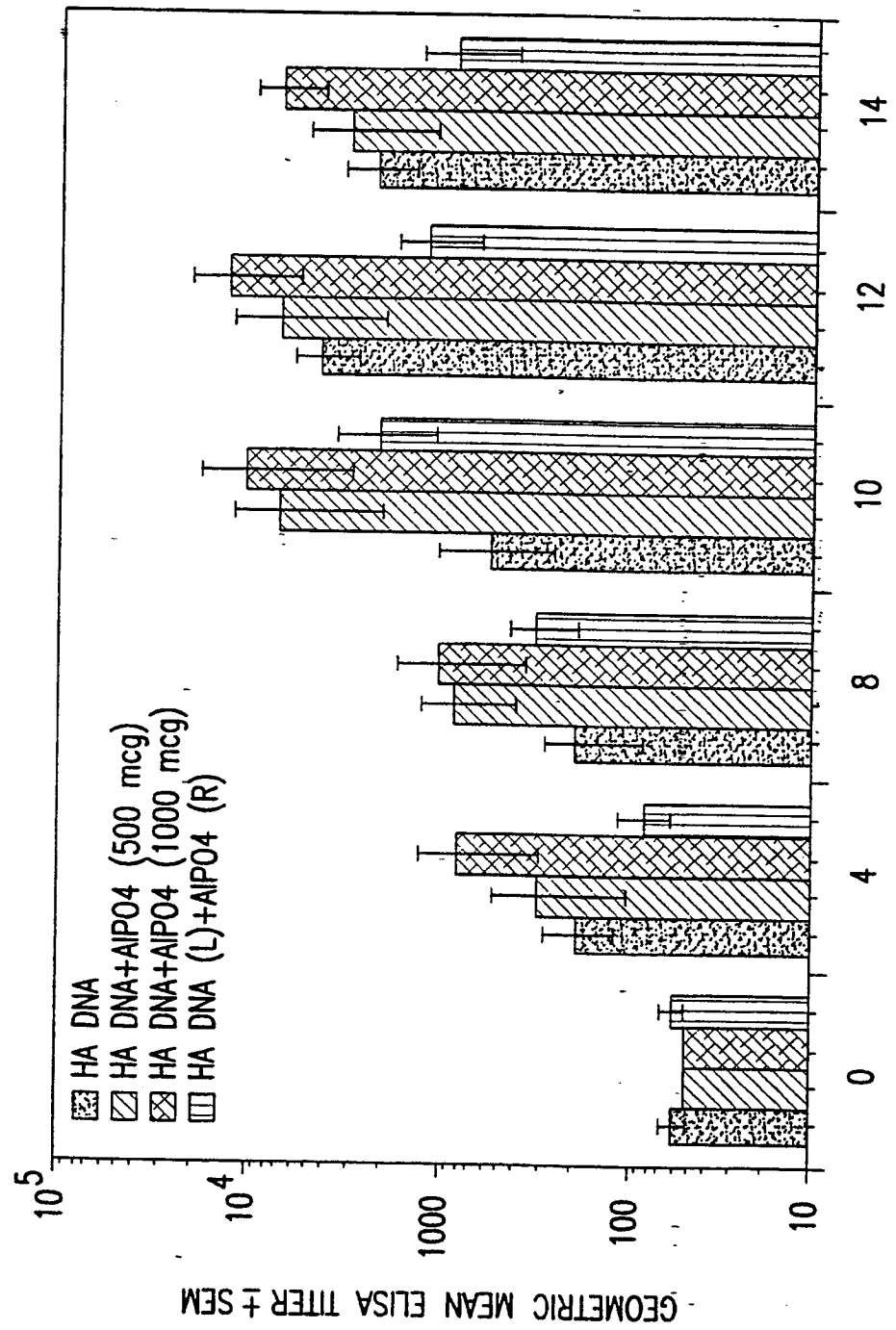
GEOMETRIC MEAN ELISA TITER ± SEM
10^5
10^4
1000
100
10
HA DNA
HA DNA+AIPO4 (500 mcg)
HA DNA+AIPO4 (1000 mcg)
HA DNA (L)+AIPO4 (R)
0
4
8
10
12
14
WEEK

FIG.12B

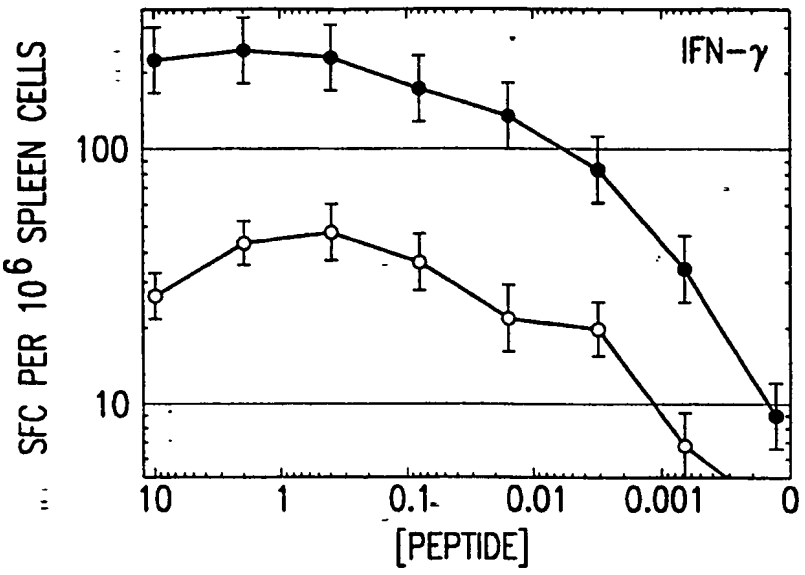

FIG.13A

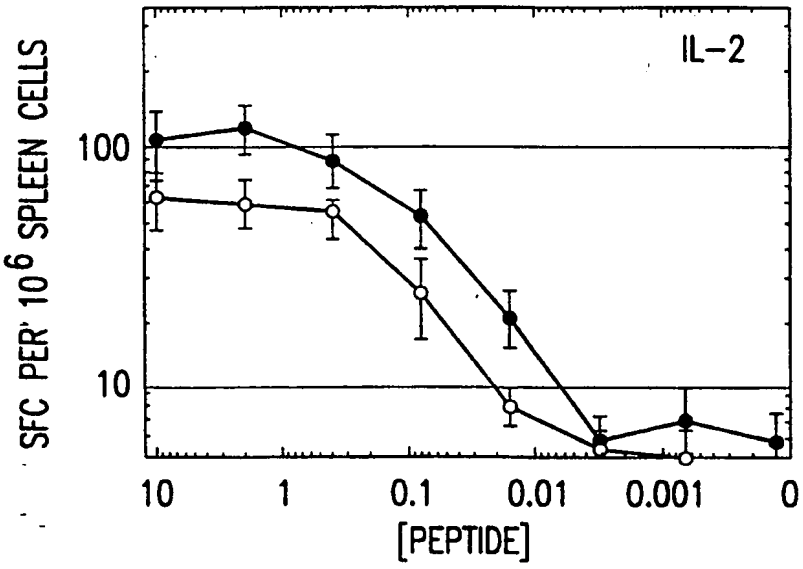

FIG.13B

SEQUENCE LISTING

<110> Merck & Co., Inc.

<120> POLYNUCLEOTIDE VACCINE FORMULATIONS

<130> 19907YIA PCT

<160> 14

<170> FastSEQ for Windows Version 3.0

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<211> 23
<212> DNA
<213> Artificial Sequence

<220>
<223> Oligonucleotide

<400> 1
ctatataagc agagctcgtt tag 23

<210> 2
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Oligonucleotide

<400> 2
gtagcaaaga tctaaggacg gtgactgcag 30

<210> 3
<211> 39
<212> DNA
<213> Artificial Sequence

<220>
<223> Oligonucleotide

<400> 3
gtatgtgtct gaaaatgagc gtggagattg ggctcgcac 39

<210> 4
<211> 39
<212> DNA
<213> Artificial Sequence

<220>
<223> Oligonucleotide

<400> 4

gtgcgagccc aatctccacg ctcattttca gacacatac 39

<210> 5
<211> 78
<212> DNA
<213> Artificial Sequence

<220>
<223> Oligonucleotide

<400> 5
gatcaccatg gatgcaatga agagagggct ctgctgtgtg ctgctgctgt gtggagcagt 60
cttcgtttcg cccagcga 78

<210> 6
<211> 78
<212> DNA
<213> Artificial Sequence

<220>
<223> Oligonucleotide

<400> 6
gatctcgctg ggcgaaacga agactgctcc acacagcagc agcacacagc agagccctct 60
cttcattgca tccatggt 78

<210> 7
<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> Oligonucleotide

<400> 7
ggtacaaata ttggctattg gccattgcat acg 33

<210> 8
<211> 36
<212> DNA
<213> Artificial Sequence

<220>
<223> Oligonucleotide

<400> 8
ccacatctcg aggaaccggg tcaattcttc agcacc 36

<210> 9
<211> 38
<212> DNA
<213> Artificial Sequence

<220>
<223> Oligonucleotide

<400> 9
ggtacagata tcggaaagcc acgttgtgtc tcaaaatc 38

<210> 10
<211> 36
<212> DNA
<213> Artificial Sequence

<220>
<223> Oligonucleotide

<400> 10
cacatggatc cgtaatgctc tgccagtgtt acaacc 36

<210> 11
<211> 39
<212> DNA
<213> Artificial Sequence

<220>
<223> Oligonucleotide

<400> 11
ggtacatgat cacgtagaaa agatcaaagg atcttcttg 39

<210> 12
<211> 35
<212> DNA
<213> Artificial Sequence

<220>
<223> Oligonucleotide

<400> 12
ccacatgtcg acccgtaaaa aggccgcgtt gctgg 35

<210> 13
<211> 9
<212> PRT
<213> Peptide

<400> 13
Thr Tyr Gln Arg Thr Arg Ala Leu Val
1 5

<210> 14
<211> 12
<212> PRT
<213> Peptide

<400> 14
Ile Pro Gln Ser Leu Asp Ser Trp Trp Tyr Ser Leu
1 5 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/15329

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :A61K 48/00
US CL :514/44; 435/320.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44; 435/320.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

BIOSIS, MEDLINE, EMBASE, CA PLUS
search terms: aluminum, phophate, vector, naked DNA, HIV, HSV, influenza, Hepatitis, HPV, tuberculosis

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X - Y	CHIANG et al. Research and Development of Adjuvants for Cell Culture Derived Japanese Encephalitis Vaccine. J. of Microbiology Immunology and Infection. March 1998, Vol. 31, No. 1, pages 58-63, see abstract on page 63.	1-3, 15, 19, 27 ----- 4-14, 16-18, 20-26, 28-40
Y	HEM et al. Accessibility of Antigen in Vaccines Produced by in situ alum Precipitation. Vaccine Research. 1996, Vol. 5, No. 4, pages 187-191, see entire article.	1-40
Y	BRUBAKER et al. Th1 associated Immune Responses to Beta-galatosidase Expressed by a Replication-Defective Herpes Simplex Virus. J. of Immunology. 15 August 1996, Vol. 157, No. 4, pages 1598-1604, see entire article.	1-40

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)		
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search 12 OCTOBER 1999	Date of mailing of the international search report 09 NOV 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer KAREN M. HAUDA Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/15329

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
1-40

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/15329

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	RINELLA et al. Elutability of Proteins from Aluminum-Containing Vaccine Adjuvants by Treatment with Surfactants. J. of Colloid and Interface Science. 01 January 1998, Vol. 197, No. 1, pages 48-56, see entire article.	1-40
Y	YORK et al. Immunomodulatory Effects of HSV2 Glycoprotein D in HSV1 Infected Mice: Implications for Immunotherapy of Recurrent HSV Infection. Vaccine. 1995, Vol. 13, No. 17, pages 1706-1712, see entire article.	1-40
Y	US 5,550,214 A (EBERLEIN et al.) 27 August 1996, paragraph bridging columns 19-20, especially lines 62-65.	1-40